

THE USE OF PCR AND T-RFLP AS A MEANS OF IDENTIFYING SOURCES OF FECAL  
BACTERIA POLLUTION IN THE TIDAL CREEKS OF NEW HANOVER COUNTY,  
NORTH CAROLINA

Mary I.H. Spivey

A Thesis Submitted to the  
University of North Carolina Wilmington in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science

Center for Marine Science

University of North Carolina Wilmington

2008

Approved by

Advisory Committee

Bongkeun Song

Parke A. Rublee

Michael A. Mallin

Chair

Accepted by

Dean, Graduate School

## TABLE OF CONTENTS

ABSTRACT .....	iii
ACKNOWLEDGEMENTS .....	iv
DEDICATION .....	v
LIST OF FIGURES .....	vi
LIST OF TABLES .....	vii
BIOGRAPHICAL SKETCH .....	viii
INTRODUCTION .....	1
MATERIALS AND METHODS.....	12
RESULTS & DISCUSSION.....	21
CONCLUSIONS.....	50
REFERENCES .....	52

## ABSTRACT

Tidal creeks are deteriorating more each day as a consequence of increased pollution caused by a boom in urbanization, which has intensified the anthropogenic impact on these ecosystems. These systems are subject to anthropogenic pollutants such as nutrients, pesticides, and fecal bacteria, which can cause loss of habitat, anoxia, and shellfish bed closures. In 2001, 13,410 recreational area closings and advisories were issued nationwide. Nearly 90% of these were due to the level of fecal coliform bacteria in the water. The standard method for fecal coliform pollution measurement enumerates, but does not distinguish between sources. Using standard methods for fecal coliform bacteria enumeration, and molecular methods of PCR and T-RFLP for bacterial source tracking using the genera *Bacteroides* as a target, it was possible to identify areas with high level of fecal coliform bacteria pollution as well as distinguish between human, canine and ruminant sources. Of the 54 samples, less than 23% were positive for canine fecal bacteria DNA by PCR, indicating fairly successful domestic animal waste management. Ruminant sources were found in 12 of the 54 PCR samples, mainly in the upstream sampling areas and near a known horse farm. Human fecal bacteria DNA was found in 18% of the PCR samples. T-RFLP produced 40 peaks, each corresponding to a bacterial taxon. Using the MiCA T-RFLP PAT+, it was possible to identify 13 of the 40 peaks, 11 of which were human-specific. The presence of human-specific DNA is of particular concern. New Hanover County has been plagued with sewer-system failures. The presence of human-borne *Bacteroides* DNA is indicative of either continued sewer-line problems, septic system failures, or a general persistence in the bacteria itself.

## ACKNOWLEDGMENTS

I would first like to acknowledge Dr. Mike Mallin, my advisor, for providing me with this opportunity and continually providing the support and guidance needed for me to achieve my goals. I would like to extend my sincere thanks to Dr. Bongkeun Song, for helping convert me to a molecular biologist and for never failing to answer my thousands of questions. My thanks also go to Dr. Parke Rublee and his laboratory for getting my feet wet and giving me my starting points. To the three of you I am eternally grateful.

This would not have been possible without the help of many people if have had the pleasure of working with at CMS over the years. I would like to thank Holly Oates-Barker, Jennifer Randolph, Jen Bagwell, and Matt Hirsch of the Microbial Ecology Laboratory for your continued support with molecular techniques. Thanks to Amanda Maness and Heather Wells for your help with GIS mapping. I would also like to thank Douglas Parsons, Ned DuRant, Kim Duernberger, Jenny Johnson, Heather Wells, Asher Williams, and Crystal Shaw; past and present members of the Aquatic Ecology Laboratory who provided hours of field and laboratory assistance. My sincere appreciation also goes to Matthew McIver, the Aquatic Ecology Lab manager and my partner in crime.

I would also like to thank my parents for always reminding me of the importance of a good education. Your help in getting here will always be remembered. Thanks as well to all of my friends and family for your love and support. You all had a part in this accomplishment.

## DEDICATION

This work is dedicated to all of those who find shelter in the water; who feel at home when they hear the rippling of the waves and see the glimmer of the sun over a horizon of blue. May you always find peace there.

## LIST OF FIGURES

Figure	Page
1. A map of the six sample areas and nine stations .....	10
2. A flow chart outlining the PCR methods used.....	17
3. A flow chart outlining the T-RFLP DNA methods used .....	20
4. Agarose gel picture of PCR products.....	27
5. T-RFLP profiles from BC-NBU for the months of August (top), June (middle), and July (bottom) 2006 .....	38
6. T-RFLP profiles from BC-SB for the months of February (top), June (middle), and July (bottom) 2006 .....	39
7. T-RFLP profiles from FC-17 for the months of January (top) and June (middle) 2006 and December (bottom) 2005 .....	40
8. T-RFLP profiles from FOY for the months of June (top) and July (bottom) 2006 ...	41
9. T-RFLP profiles from HW-DT for the months of June (top), July (second), and August (bottom) 2006 and December (third) 2005.....	42
10. T-RFLP profiles from HW-GP for the months of July (top) and June (bottom) 2006 and December (middle) 2005.....	43
11. T-RFLP profiles from NB-GLR for the months of February (top) and June (third) and July (bottom) 2006, and December (second) 2005 .....	44
12. T-RFLP profile from PC-BDDS for the month of January 2007 .....	45
13. T-RFLP profiles from WC-MB for the months of January (top) and June (bottom) 2006.....	45
14. T-RFLP profiles from Hewletts Creek stations SB-PGR (top), HC-3 (second), NB-GLR (third) and MB-PGR (bottom) for the November 2006 sewage spill .....	46
15. A principal component analysis comparing the physical properties, nutrients, and chlorophyll <i>a</i> concentrations to host-specific <i>Bacteroides</i> group percentages .....	47

## LIST OF TABLES

Table	Page
1. The nine sampling stations, divided by creek.....	10
2. Host-specific primers used in this study .....	14
3. PCR mixtures and thermocycler parameters for each reaction.....	16
4. The physical parameters, nutrients, and chlorophyll <i>a</i> concentrations for each month sampled at each of the nine stations .....	22-23
5. Monthly fecal coliform bacteria counts at each station. Bolded entries are samples that were taken during rain events .....	25
6. PCR detection results.....	28
7. Host-specific <i>Bacteroides</i> T-RFLP peaks present in each DNA fingerprinting sample, and what percentage of the sample they account for.....	32-34
8. Host-specific <i>Bacteroides</i> group percentages based on T-RFLP results .....	36
9. Linear regression analysis results comparing physical parameters, nutrients, and chlorophyll <i>a</i> concentrations to host-specific group percentages. Presented as Pearson product-moment correlation coefficient ( <i>r</i> ) and probability ( <i>P</i> ) .....	49

## BIOGRAPHICAL SKETCH

Mary Spivey was born on December 31, 1979 in Rockingham, NC. She became interested in marine science in middle school through science programs for the academically gifted. Upon graduation from Richmond Senior High School in 1998, she began a work on a Bachelor's Degree in Biological Sciences from North Carolina State University, where she completed undergraduate honor's research under the direction of Dr. JoAnn Burkholder and Dr. Brant Touchette at N.C. State's Center for Applied Aquatic Ecology. Her undergraduate research led her to continue her interest in aquatic ecology with Dr. Michael Mallin's Aquatic Ecology Laboratory at the University of North Carolina Wilmington Center for Marine Science. Upon graduation in December 2008, Mary plans to continue her work at the Center for Marine Science.



## INTRODUCTION

### **Tidal Creek Ecosystems**

Tidal creeks are shallow, tidally influenced estuaries that are abundant in low-energy coastal areas. Tidal variation is very important in these ecosystems, as it provides a means of transportation for some organisms, a method of food delivery for sessile creatures, and a means of sediment transport for the entire system (Mallin et al. 1999). Because tidal creeks are so numerous in coastal areas, and their surface-to-volume ratio is so large, their influence on numerous ecological processes may be greater than that of larger estuaries (Dame et al. 2000). These areas and the surrounding marshes are also very important nursery areas for fish and shellfish. Tidal creeks provide habitat and food to many commercially important species. Despite all of these important processes that tidal creek ecosystems facilitate, the ecological importance of these systems is often overshadowed by the lack of research in comparison to more expansive and conspicuous estuarine systems, such as the Chesapeake Bay (Mallin and Lewitus 2004).

### **The Problem with Tidal Creeks**

Tidal creek ecosystems are suffering more each day as a consequence of increased urbanization. Tourists and potential residents flock to coastal areas to have use of public waterways for swimming, shellfishing, finfishing, and other water activities. Because tidal creek ecosystems are aesthetically beautiful and recreationally appealing, more and more people relocate to these areas every year. This rise in urbanization has intensified the anthropogenic impact on these ecosystems (Mallin et al. 2000). The clearcutting and development associated with urbanization decrease the density of vegetation adjacent to the tidal creeks, which increases both the velocity and the volume of stormwater entering the creek (Mallin and Lewitus 2004).

This stormwater runoff often contains bacteria or other pollutants. When runoff flows through vegetated buffers, it reaches surface waters much more slowly and in a much cleaner state than stormwater that flows through areas of dense impervious surface coverage (Mallin et al. 2000).

Anthropogenic impacts on tidal creek ecosystems include eutrophication, turbidity and siltation, and shellfish bed closures due to fecal coliform pollution. Sources of nutrient and/or fecal bacteria pollutants can include fertilizers, septic system leachate, leaking sewer mains, wild and domestic animal wastes, and overland runoff. In South Carolina alone, pollutants have resulted in the closing of one-third of the shellfish harvesting areas because of the potential health risk to humans (Webster et al. 2004). In the Florida Keys, human waste has been linked to a high presence of enteric viruses in the waters and resulted in the documentation of a swimmers' risk in the area (Griffin et al. 1999, Nobles et al. 2000). In 2001, 13,410 recreational area closings and advisories were issued nationwide. Nearly 90% of these closings were due to the level of fecal coliform bacteria in the water (Olyphant 2003). Since humans are more likely to catch a human-borne illness, anthropogenic fecal coliform sources are of special concern (McLellan and Jensen 2003). For tidal creeks, the potential for both shellfish bed closures and human contact risk from fecal-polluted waters points toward the need for better understanding and management of these waters.

### **Fecal Coliform Bacteria Pollution**

The increased presence of fecal matter pollution in tidal creek areas has been associated with the aforementioned boom in urbanization. Fecal presence can be detected by measuring the level of fecal coliform bacteria in the water. Fecal coliform bacteria live in the intestines of warm-blooded organisms. These bacteria are commonly used as indicators of the presence of pathogens in waters affected by polluted inputs (Mallin 2000). Though fecal coliform bacteria

are not harmful in their own right, they indicate the presence of human and/or animal waste that could introduce other harmful bacteria into the water source (Kim et al. 1996). The risk for recreational water users to contract a communicable waterborne disease is increased in bodies of water that are impaired by fecal matter pollution (Canale et al. 1993).

In order to understand and manage fecal bacteria pollution in any body of water, one must first be able to identify the source of the pollution (Kelsey et al. 2004). Anthropogenic point sources can include septic systems and sewage treatment plants. These pollutants come from a centralized location or an outfall pipe, so their source is known. Non-point sources can be sediments, manure, and stormwater runoff, just to name a few. These pollutants enter the creek from sources that are not readily diffusible. The most significant source of non-point source pollution in urban watersheds is runoff (Marsalek and Rochfort 2004). Coastal development has increased both point and non-point runoff (Mallin et al. 2000). Transport mechanisms can include direct discharge, groundwater flow, runoff, and drainage systems (Jamieson et al. 2003). An increasing number of research studies are finding that there is a correlation between land-use and development and the concentration of coliform units in the nearby watersheds (Kelsey et al. 2004). In order to meet set water quality goals in urban watersheds, advanced knowledge and understanding of non-point source pollution is necessary (Marsalek and Rochfort 2004).

A link has also been noted between fecal coliform counts and high levels of rainfall. Young and Thackston (1999) reported that fecal coliform bacteria counts were very high during rainfall events. They further noted that the high bacteria counts were likely due to stormwater runoff. Kelsey et al. (2004) supported this finding by suggesting the only complete model for regulating shellfish bed closure in Murrells Inlet, SC would include a 48-hour rainfall measurement. Sewage and stormwater overflows associated with rainfall events, combined with the discharge

of wastewater from treatment plants, are considered to be most important influences on the overall quality of shellfish harvesting waters (Burkhardt et al. 1999).

Landscape changes associated with increased development, including clearcutting and impervious surface coverage, can increase the concentration of bacterial colonies making their way into area waters by increasing the volume of stormwater running into the watershed (Mallin et al. 2000). Research has found that stormwater runoff from urbanized areas had very high levels of fecal coliform bacteria compared to rural areas (Gannon and Busse 1989). The density of fecal coliform bacteria in local tributaries in Nashville, TN was found to be directly related to the population, housing density, development, percent impervious cover, and potential domestic pet waste in nearby neighborhoods (Young and Thackston 1999). Mallin et al. (2000) found a strong positive correlation between the percent impervious cover and the density of coliform units in several North Carolina estuarine ecosystems. Subsequently, Holland et al. (2004) found a similar relationship in tidal creeks around Charleston, SC. Since human development has been linked to an increased presence of fecal coliform in the local waters, human consumption of, or recreation involving, contaminated water may help to spread disease (Entry and Farmer 2001). Coastal areas are particularly vulnerable to these problems because of the steady increase in population and tourism and the concentration of recreational water sources in these areas (Mallin et al. 2001).

Agricultural operations have also been found to be a source of fecal coliform pollution (Mallin 2000). Concentrated animal feeding operations (CAFOs) have had problems with their waste management practices. Swine production wastes are fed into management lagoons. The contents of these lagoons are sometimes sprayed onto crop fields as a fertilizer. Through groundwater seepage, wastewater lagoon overflows, and/or stormwater runoff, surrounding

waterways or groundwater sources may be contaminated by fecal coliform bacteria. In 1995, 22 miles of the New River in North Carolina was polluted when a waste lagoon ruptured and spilled 25 million gallons of swine waste into the river waters (Mallin and Cahoon 2003). Such problems are more common in a state like North Carolina, where hogs outnumber people and hurricanes help to escalate CAFO wastewater runoff (Mallin 2000). This is even more frightening when one considers that fecal bacteria can survive six months or longer in groundwater environments, possibly rendering the water unfit to drink (Conboy and Goss 2001). Hurricanes are often a catalyst for pollution due to extensive amounts of property damage and large quantities of rainfall in short periods of time. Hurricane Floyd alone was responsible for 13 reported CAFO lagoon floodings in the Cape Fear River area (Mallin et al. 2002).

There are some steps/actions that minimize the risk of water contamination, especially with respect to human pathogens. Since the testing for specific pathogens is difficult and expensive, the use of indicator organisms has become a popular solution (Jamieson et al. 2003). Typically, members of the genera *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* are used to indicate the presence of coliforms. *Escherichia coli* (*E. coli*) is a standard indicator organism used to determine the presence of fecal contamination. The method involves filtering samples from a watershed and growing fecal coliforms to obtain an estimate of the number of colony forming units (CFUs) per unit volume (APHA, AWWA, and WEF Method 9221 E. 1998, Field et al. 2003). Widespread use of this method has allowed for the development of water quality standards in reference to human usage. The national standard for shellfishing waters is 14 CFUs per 100mL of water of fecal coliform bacteria (USFDA 1995). Shellfish may not be harvested in areas where the fecal coliform measurements exceed this amount. Individual states decide their own standards for recreational human contact. North Carolina's standard is 200 CFUs per

100mL of water of fecal coliform bacteria for freshwater systems, based on the geometric mean of at least five consecutive samples taken during a 30-day period. The count is not to exceed 400 CFUs in more than 20 percent of samples during the same period (NCDENR 2005).

### **Identifying Nonpoint Sources of Fecal Bacteria Pollution**

While the standard method for fecal coliform bacteria measurement offers a fairly accurate look at the level of fecal pollution in a given watershed, it does not distinguish between the many possible warm-blooded sources of the pollution. If one wishes to identify the exact source of fecal pollution, the use of *E. coli* does not easily allow for the separation of human and animal origins (Conboy and Goss 2001). A complete assessment of the human health risk and development of better watershed management practices requires a method that can pinpoint the sources of contamination (Field et al. 2003). Griffin et al. (1999) argued that the Florida Keys would require a better understanding of transport, abundance, and settling of microbial pollutants, and their resulting health risks, in order to improve the wastewater management systems.

Some source identification research has been done using multiple antibiotic resistance analysis as a means for separating human and animal sources of *E. coli* (Parveen et al. 1997). Since humans, domestic animals, and livestock receive antibiotic treatments, the bacteria from livestock and human sources have been found to be more resistant to the antibiotics (Guan et al. 2002). This method involves growing fecal streptococcal isolates in large quantities and using numerous antibiotics on these cultures to evaluate resistance. It requires intensive labor, is very time-consuming, and is often inaccurate (Field et al. 2003).

Conboy and Goss (2001) recognized the need for an organism that is unique to animals or humans to use as a more specific indicator of the sources of fecal pollution. Their research

focused on using *Clostridium perfringens* (*C. perfringens*) as an indicator of domestic livestock manure. They found, however, that while *C. perfringens* is a good indicator of animal fecal pollution, it is not unique to domestic livestock. Conboy and Goss (2001) concluded that, when testing for fecal coliform sources, it is best to use an assemblage of indicator bacteria to cover the number of potential animals of origin.

Bernhard and Field, two researchers at Oregon State University, published two papers outlining a new method for identifying nonpoint sources of fecal pollution. Their research involved using *Bacteroides* as indicators because of their abundance in warm-blooded intestinal tracts and because they are not sensitive to salinity like fecal coliforms. Bernhard and Field (2000a) used molecular techniques known as length heterogeneity polymerase chain reaction (LH-PCR), and terminal restriction fragment length polymorphism (T-RFLP), to identify host-specific patterns that allowed them to pinpoint DNA fragment lengths that were unique to cows or humans. They amplified both human and cow fecal DNAs with primers specific for the *Bacteroides-Prevotella* species and *Bifidobacterium* species, and then separated the fragments by size using GeneScan software with an ABI DNA sequencer. Through these methods, they were able to identify one human-specific and two cow-specific markers from fecal 16s rDNA sequences that corresponded to *Bacteroides-Prevotella* markers. They used this information to develop human and cow-specific DNA primers. Their methods allowed them to detect host-specific markers in water pollutant concentrations of  $2.8 \times 10^{-5}$  to  $2.8 \times 10^{-7}$  g (dry weight) of feces/liter and  $6.8 \times 10^{-7}$  g (dry weight) of sewage/liter (Bernhard and Field 2000a). Bernhard and Field (2000a) also sampled freshwater and saltwater systems and were able to recover these markers from such samples. They used the information that they found to identify additional

sequences that allowed them to expand the cow-specific markers and primers to include all ruminant animal species (Bernhard and Field 2000b).

Dick et al. (2005) used a molecular method known as subtractive hybridization to identify dog-specific fecal markers from *Bacteroidales* species 16s rRNA gene fragments. After sequencing, these fragments were used to develop a dog-specific fecal DNA primer as well. These researchers subsequently tested the species-specific markers against other known samples to rule out any possibility that the DNA primers developed from their findings would amplify anything other than their target DNA segments. It is the primers that these researchers developed and published in Bernhard and Field (2000a and 2000b) and Dick et al. (2005) that are being used in this study. The goal of this study is to identify sources of fecal bacteria pollution using described methods.

## **Objectives**

- 1) Monitor physical, chemical, and biological parameters of water quality
- 2) Identify sources of microbial contamination using molecular techniques
- 3) Identify problems and suggest better watershed management practices for all of the tidal creeks in New Hanover County, North Carolina

## **Hypotheses**

All of the sampling stations were a part of the New Hanover County Tidal Creeks Program regular sampling regime that was run by the University of North Carolina Wilmington (UNCW) Aquatic Ecology Laboratory from 1993 through 2007. This laboratory at the UNCW Center for Marine Science (CMS) published annual water quality reports based on the findings of this program (<http://www.uncw.edu/cmsr/aquaticecology/laboratory>). The program is now managed by Coastal Planning and Engineering (<http://www.coastalplanning.net>). New Hanover County has been experiencing rapid urbanization and a population increase. Taking into account what



was already known about the sampling areas and the effect that the urbanization is continuing to have on these areas, the following hypotheses were proposed:

- H<sub>1</sub>:** The presence of general *Bacteroides* will be found at all nine of the sampling stations because all six of these tidal creeks have experienced warm-blooded fecal bacteria pollution.
- H<sub>2</sub>:** The presence of canine species will be found at all nine of the sampling stations because New Hanover County has a very large population of domestic animals.
- H<sub>3</sub>:** The presence of ruminant species, which include cow, deer, goat, horse and sheep species, will be found at the less urbanized sampling stations, or those stations that are found upstream near the headwaters of the creeks. These stations, namely, would be: FC-17, FOY, HW-DT and HW-GP. If ruminant sources are found, presumably deer or horses would be the sources
- H<sub>4</sub>:** The presence of human species will not be found at any of the nine sampling stations. All of these areas have either public sewer lines or septic systems. Provided that there are overflow or infrastructure failures, the presence of human species should not be detected at any station.

### **Sample Areas**

The sample areas are six tidal creeks that are all located in southeastern North Carolina (Mallin et al. 2001). They include Bradley, Futch, Hewletts, Howe, Pages, and Whiskey Creeks. Figure 1 is a map displaying the location of all sample areas and stations.

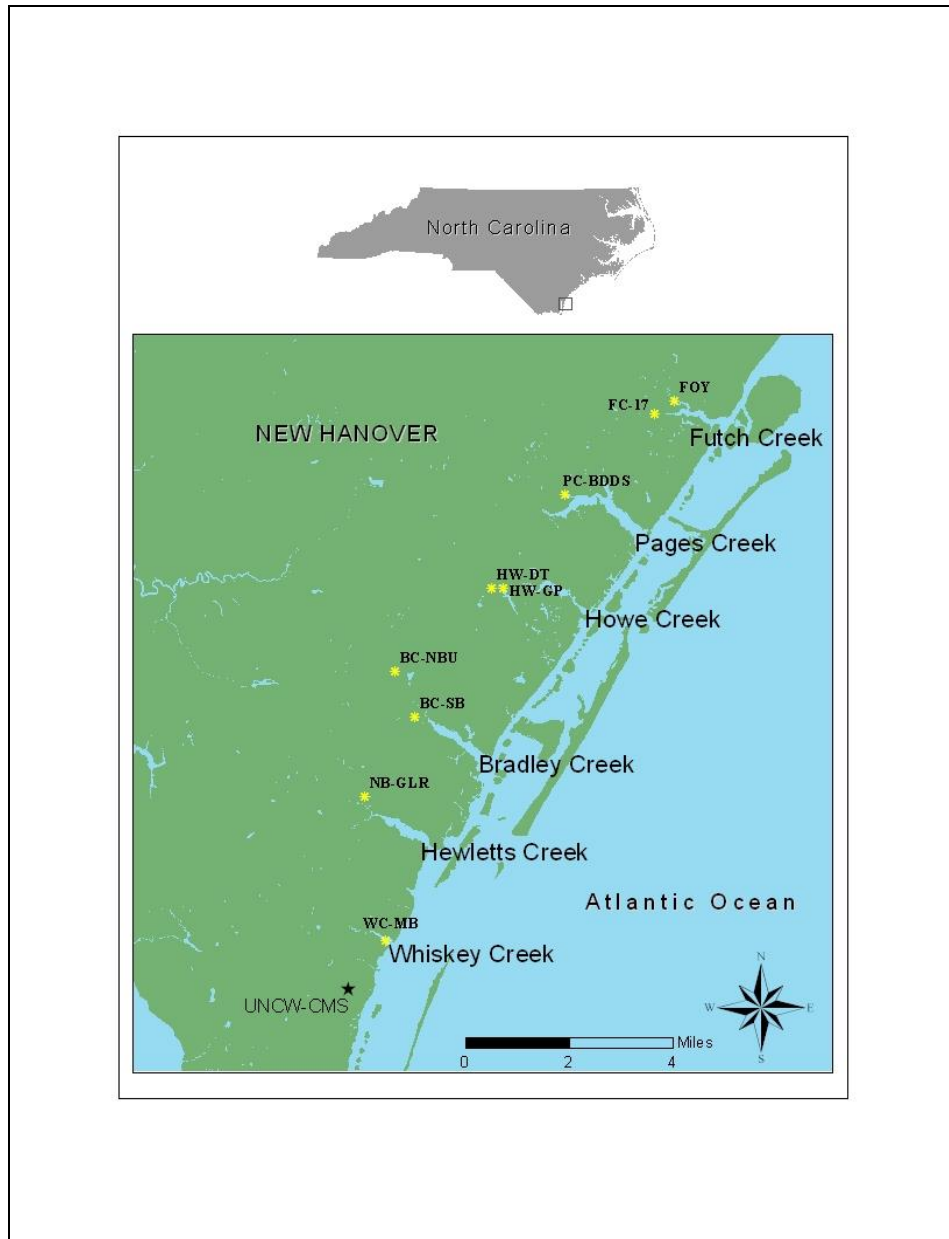


Figure 1: A map of the six sample areas and nine stations. Map courtesy of H. Wells and A. Maness.

Nine stations among the six creeks were sampled as part of this research (Table 1).

Table 1: The nine sampling stations, divided by creek.

<b>Bradley</b>	<b>Futch</b>	<b>Hewletts</b>	<b>Howe</b>	<b>Pages</b>	<b>Whiskey</b>
BC-NBU	FC-17	NB-GLR	HW-DT	PC-BDDS	WC-MB
BC-SB	FOY		HW-GP		

## Site Description

**BC-NBU:** This station is in the headwaters of the northern branch of Bradley Creek. It drains an area of Wilmington that is highly urbanized. N 34 13.959, W 77 51.141

**BC-SB:** This station is located on Wrightsville Avenue in Wilmington and drains the headwaters of the southern branch of Bradley Creek. There is a horse farm just upstream from the station. N 34 13.186, W 77 50.747

**FC-17:** This station is the uppermost station that is sampled on Futch Creek. It drains the headwaters of Futch Creek, which is considered to be the least urbanized tidal creek in New Hanover County. Many residents of Futch Creek still operate on septic systems instead of the county sewer system. N 34 18.220, W 77 45.825

**FOY:** This station is on the northern branch of Futch Creek, a relatively pristine area on the border of New Hanover and Pender Counties. N 34 18.425, W 77 45.422

**HW-DT:** This station is the uppermost station that is sampled on Howe Creek. This station receives drainage from parts of Landfall (residential golf course), Demerest Landing (residential area), and the Mayfaire (large shopping and residential area) developments. It is subject to stormwater runoff from these developments. N 34 15.327, W 77 48.918

**HW-GP:** This station is about 0.5km downstream from HW-DT and receives more drainage from Landfall through the Graham Pond catchment basin. N 34 15.337, W 77 49.171

**NB-GLR:** This station is on the northern branch of Hewletts Creek, where it passes under Greenville Loop Road in Wilmington. It drains a highly urbanized area and is downstream from a dog boarding and grooming facility. N 34 11.870, W 77 51.790

**PC-BDDS:** This station is in the headwaters of Pages Creek. It receives drainage from an urbanized area. Pages Creek, in general, is a relatively well-flushed estuary. N 34 16.886, W 77 47.650

**WC-MB:** This station is at a boat-yard at the mouth of Whiskey Creek, an urbanized tidal creek. The boat-yard is a closed-head facility that has year-round, live-aboard residents. N 34 09.449, W 77 51.384

## MATERIALS AND METHODS

### **Sample Collection, Filtration, and Storage**

Monthly samples were collected from each station except PC-BDDS during the months of December 2005 and January, February, June, July and August 2006. Monthly samples from PC-BDDS were collected from January to June, 2007. Water samples were collected at each station for chlorophyll *a* analysis, fecal coliform counts, and DNA extraction. Physical characteristics of the water at each station were measured using a YSI 6920 multi-parameter water quality sonde (i.e. water temperature, salinity, conductivity, pH, turbidity, and dissolved oxygen).

Chlorophyll *a* samples were collected just below the surface of the water using three brown 150mL Nalgene bottles per station. These samples were transported on ice and filtered upon return to the lab. Each bottle was shaken and 50mL of the sample was filtered through a 25mm Whatman glass fibre filter. The triplicate samples were wrapped in aluminum foil and stored and -20°C until they could be processed.

The water samples for DNA analysis were collected in autoclaved 500mL Pyrex glass bottles. The samples were transported on ice and allowed to sit no longer than six hours before filtration. Upon returning to the lab, the samples were filtered using autoclaved glassware and sterile Whatman GF/F 47mm filters, with a nominal pore size of 0.7µm. From the samples collected in the winter months (December 2005, January and February 2006), 100mL of sample was filtered for each station. In the month of June 2006, this amount was increased to 500mL in order to obtain a more concentrated sample. Due to the amount of suspended materials that most of these samples contained, 500mL was found to be too difficult to filter. During the months of July and August 2006, this amount was decreased to a more manageable 400mL. After filtration, the

filters were wrapped individually in aluminum foil and stored at  $-20^{\circ}\text{C}$  until DNA extraction from the filter could be performed.

The fecal coliform samples were collected in autoclaved 500mL Pyrex glass bottles. The samples were transported on ice and were held for no longer than six hours before filtration.

### **Chlorophyll *a* Analysis**

To determine the amount of chlorophyll *a* present in each sample, the filters were steeped in 10mL of 90% acetone and refrigerated at  $4^{\circ}\text{C}$  for 18 to 24 hours. Samples were then allowed to warm to room temperature and their fluorescence was measured using a fluorometer at an excitation wavelength of 430nm and an emission wavelength of 663nm. A mean was determined and reported in  $\mu\text{g/L}$  (APHA, AWWA, and WEF Method 10200 H. 1998).

### **Mean Fecal Coliform Determination**

Samples were filtered following APHA, AWWA, and WEF Method 9221 E. (1998) using autoclaved glassware and sterile Millipore white gridded 47mm filters, with a pore size of  $0.45\mu\text{m}$ . All samples were filtered at a volume of 100mL. Some were filtered at a volume of 10mL to avoid a density of colonies on the filter that would be too numerous to count (TNTC). These filters were placed in sterile petri dishes with pads soaked in mean fecal coliform (MFC) broth media, composed of 3.7g of MFC medium, 100mL of distilled water, and 1mL of Rosolic Acid solution (0.1g Rosolic Acid crystals dissolved in 10mL of 0.2N NaOH). The plates were then sealed in plastic storage bags and incubated in a water bath at  $44.5^{\circ}\text{C}$  for 23 to 25 hours. The plates were then removed from the water bath and the number of blue colonies on the gridded filter were counted using a stereoscope and clicker. The counts were recorded as the number of CFUs/100mL of water.

## DNA Extraction

The DNA was extracted using the PowerSoil™ DNA Isolation Kit from MO BIO Laboratories, with the protocol slightly modified for extraction from a filter instead of a soil sample. A portion of the filter was ground using a PowerBead Tube and tissue grinder, and then the extraction was completed per manufacturer's instructions. The MO BIO PowerSoil™ DNA Isolation Kit uses a detergent to lyse the cells and release the DNA, and then uses several solutions to help precipitate materials that may reduce the purity of the DNA (such as non-DNA humics, cell debris, and proteins) and inhibit PCR reactions.

## PCR Detection Methods

A list of all of the primers that were used in this study can be found in Table 2. The 27F and 1522R primers were paired together to amplify universal 16s rDNA, which is the DNA region used for identifying bacteria. All of the other forward primers were paired with the Bac708R reverse primer.

Table 2: Host-specific primers used in this study.

Primer	Sequence (5'-3')	Target	Specificity	Reference
27F	AGAGTTTGATCMTGGCTCAG	16s rRNA gene	Bacteria	Suzuki and Giovannoni (1996)
1522R	AAGGAGGTGATCCANCCRCA	16s rRNA gene	Bacteria	Suzuki and Giovannoni (1996)
DF475F	CGCTTGATGTACCGGTACG	SHDogf	Canine	Dick et al (2005)
HF183F	ATCATGAGTTCACATGTCCG	HF8 cluster, HF74	Human	Bernhard and Field (2000b)
CF193F	TATGAAAGCTCCGGCC	CF151 cluster	Ruminant	Bernhard and Field (2000b)
Bac32F	AACGCTAGCTACAGGCTT	<i>Bacteroides-Prevotella</i>	General <i>Bacteroides</i>	Bernhard and Field (2000a)
Bac708R	CAATCGGAGTTCTTCGTG	<i>Bacteroides-Prevotella</i>	General <i>Bacteroides</i>	Bernhard and Field (2000a)

All of the samples were first amplified with the universal 16s primers. These PCR products were then used as template for all PCR reactions for each sample. This process is known as nested PCR. It reduces the potential contamination that can be caused by the primers binding to unspecified sites, as it is unlikely that non-target DNA would be amplified by two separate sets of primers.

### PCR Mixtures and Conditions:

The recipe for each 16s universal primer PCR mixture was as follows: each 25 $\mu$ L mixture contained 1 unit of *Taq* polymerase (0.5 $\mu$ L), 1 $\mu$ L of each of the primers 27F and 1522R, at a concentration of 0.4 $\mu$ M each, 0.2 $\mu$ L of 10M deoxynucleoside triphosphates (dNTPs), 2.5 $\mu$ L of 25mM MgCl<sub>2</sub>, and 2.5 $\mu$ L of 10x PCR buffer. The template was added to the mixture at a volume of 1 $\mu$ L and the mixture was then brought up to 25 $\mu$ L using sterile deionized water. Thermocycler parameters were as follows: an initial 5-minute denaturation at 95°C, followed by 35 cycles, each consisting of a denaturation at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension at 72°C for 2 minutes.

The recipe for the general *Bacteroides* primer PCR mixture was as follows: each 25 $\mu$ L mixture contained 0.5 $\mu$ L of *Taq* polymerase, 1 $\mu$ L of each of the primers Bac32F and Bac708R, each at a concentration of 10 $\mu$ M, 0.2 $\mu$ L of 10x dNTPs, 2.5 $\mu$ L of MgCl<sub>2</sub>, and 2.5 $\mu$ L of *Taq* polymerase buffer. The products of the 16s universal primer PCR reaction were used as the template, creating a nested PCR reaction. The template was added to the mixture at a volume of 1 $\mu$ L and the mixture was then brought up to 25 $\mu$ L using sterile deionized water. Thermocycler parameters used were the same as the 16s universal primer reaction.

The mixtures for each of the canine, human, and ruminant PCR reactions were the same as the recipe for the general *Bacteroides* mixture, using only different forward primers required for each target as shown in Tables 2 and 3. The reverse primer, Bac708R, remained the same for each of these reactions as it works universally with all of the different forward primers listed in Table 2, aside from the 27F primer. All primers were used at a concentration of 10 $\mu$ M. The thermocycler parameters were also the same as those for the 16s universal primer PCR reaction.

All of the mixtures and thermocycler parameters for each PCR reaction are outlined in Table 3.

All of the PCR reactions were run with a positive and negative control.

Table 3: PCR mixtures and thermocycler parameters for each PCR reaction.

Primer	Taq	MgCl <sub>2</sub>	Buffer	Primers	Denaturation	Annealing	Extension
<b>16s</b>	1µL	2.5µL	2.5µL	1µL 27F, 1µL 1522R	94°C for 30 sec	55°C for 30 sec	72°C for 2 mins
<b>Bac</b>	1µL	2.5µL	2.5µL	1µL Bac32F, 1µL Bac708R	94°C for 30 sec	55°C for 30 sec	72°C for 2 mins
<b>Canine</b>	1µL	2.5µL	2.5µL	1µL DF475F, 1µL Bac708R	94°C for 30 sec	55°C for 30 sec	72°C for 2 mins
<b>Human</b>	1µL	2.5µL	2.5µL	1µL HF183F, 1µL Bac708R	94°C for 30 sec	55°C for 30 sec	72°C for 2 mins
<b>Ruminant</b>	1µL	2.5µL	2.5µL	1µL CF193F, 1µL Bac708R	94°C for 30 sec	55°C for 30 sec	72°C for 2 mins

DNA fragments that have been amplified by PCR can be separated by gel electrophoresis, which allows us to separate fragments by size (Klug and Cummings, 1997). The PCR products are run on a 1% agarose gel to separate the fragments. Gels are run with a DNA ladder, which provides a reference against which the resultant bands can be measured for length (in base pairs). The 16s fragment has a size of 1495 basepairs, the *Bacteroides* fragment is 676 basepairs, the canine fragment is 233 basepairs, the human fragment is 525 basepairs, and the ruminant fragment is 515 basepairs. For the purposes of this study, any resultant band of the correct size in the agarose gel indicated that target DNA was obtained from the sample in question. A schematic outline of the PCR methods used for this study is shown in Figure 2.



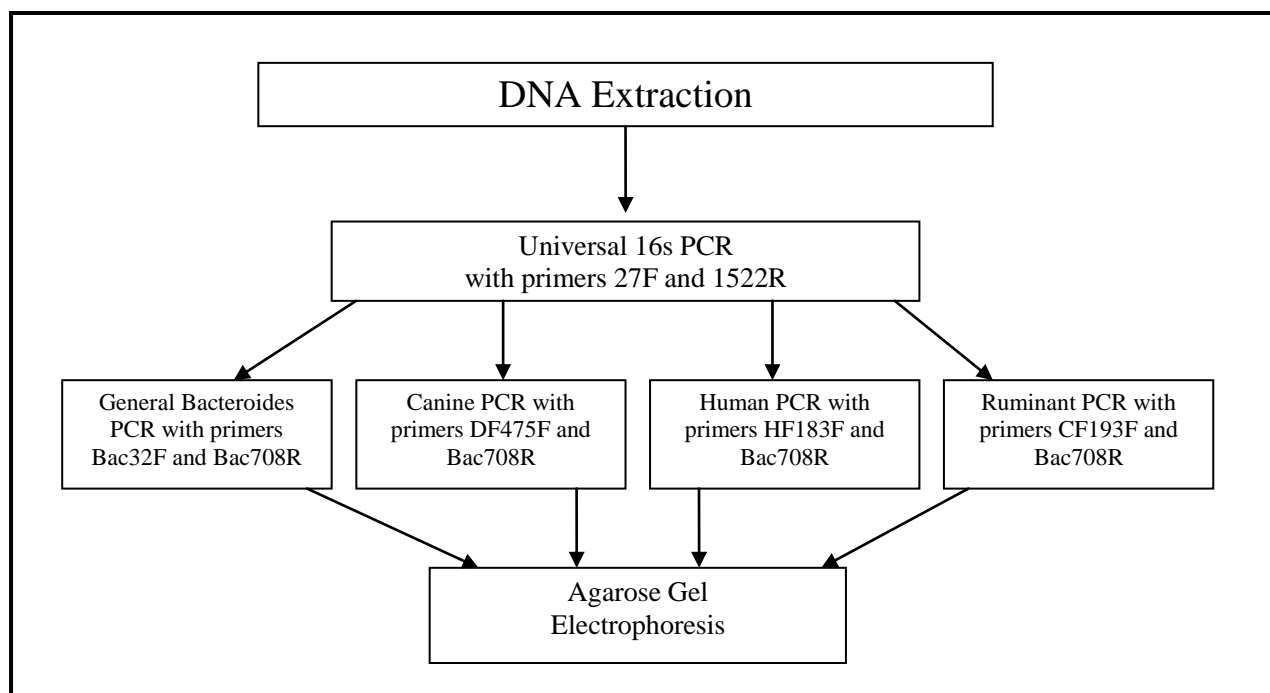


Figure 2: A flow chart outlining the PCR methods used.

### T-RFLP

In an effort to gain a more complete understanding of the bacterial communities present in each sample, and to confirm the results obtained by the direct PCR methods, PCR mixtures for select samples were also setup with a Bac32F/Bac708R primer pair, in which the forward primer was labeled with fluorescent tags (FAM labeled). The PCR mixture for these samples contained 12.5µL of Fisher Promega's GoTaq® Green Master Mix and 1µL of each primer, at a concentration of 0.4µM. The template was added at a volume of 1µL, and the reaction was then brought up to a 25µL volume using sterile deionized water. These PCR products were then run on a 1% agarose gel to determine the presence or absence of the target segment of DNA and to separate the positive bands and cut them from the gel. A GENECLAN® Turbo Kit from Q-BIO gene was then used to purify these cut fragments.

In order to determine the concentration of DNA in these samples, a Quant-iT™ DNA HS Assay from Invitrogen was used. The samples were read using a Qubit fluorometer, which

provides a fluorescence measurement used in determining the concentration of DNA in the sample. The resultant information about the concentration of DNA present in each sample was then used to determine the needed formula for enzyme digestion. For samples with  $<1\text{ng}/\mu\text{L}$  of DNA, the entire remaining volume of the sample ( $29\mu\text{L}$ ) was combined with  $3.5\mu\text{L}$  of buffer,  $0.5\mu\text{L}$  of *AciI* enzyme, and  $2\mu\text{L}$  of sterilized water to bring the sample to a final volume of  $35\mu\text{L}$ . For samples with  $>1\text{ng}/\mu\text{L}$  of DNA, an appropriate sample volume was added so that the enzyme digestion mixture contained  $20\text{ng}$  of DNA. For example, with a sample that had a concentration of  $8\text{ng}/\mu\text{L}$  of DNA,  $2.5\mu\text{L}$  of the sample was added to the enzyme digestion mixture. This was combined with  $3.5\mu\text{L}$  of buffer and  $0.5\mu\text{L}$  (5 units) of *AciI* enzyme. Sterilized water was then added to bring the final volume of the mixture to  $35\mu\text{L}$ . These reactions were incubated overnight at  $37^{\circ}\text{C}$  for digestion to occur.

The following day, these enzyme digestion mixtures received  $70\mu\text{L}$  of 75% isopropanol and they were incubated on ice at  $4^{\circ}\text{C}$  for 15 minutes. The samples were then centrifuged for 15 minutes, the alcohol was aspirated from the samples, and they were dried at  $95^{\circ}\text{C}$  to remove any excess alcohol. A  $10\mu\text{L}$  mixture of HiDi formamide and GS 500 Rox was added, the samples were incubated at  $95^{\circ}\text{C}$  for one minute, and then incubated on ice at  $4^{\circ}\text{C}$  for an additional minute. They were then vortexed and centrifuged again, and the entire  $10\mu\text{L}$  of each sample was loaded into a well on a tray for DNA fingerprinting. DNA fingerprinting was conducted on an ABI PRISM® Genetic Analyzer, from Applied Biosystems.

Upon completion of fingerprinting, each sample was represented by a profile. The profiles have representative peaks for each bacterial taxon present in the samples. The size of each fragment present is indicated in base pairs, and these fragments can be matched to a database of known fragments of DNA to identify what is present. This analysis was completed using the

Microbial Community Analysis 3 (MiCA) T-RFLP Analysis Phylogenetic Assessment Tool (PAT+) found at <http://mica.ibest.uidaho.edu/pat.php>. A pictorial representation of the methods used for the T-RFLP part of the study is shown in Figure 3.

Statistical analyses, including principal component analysis and regression, were conducted using Caneco for Windows® and Microsoft Excel.

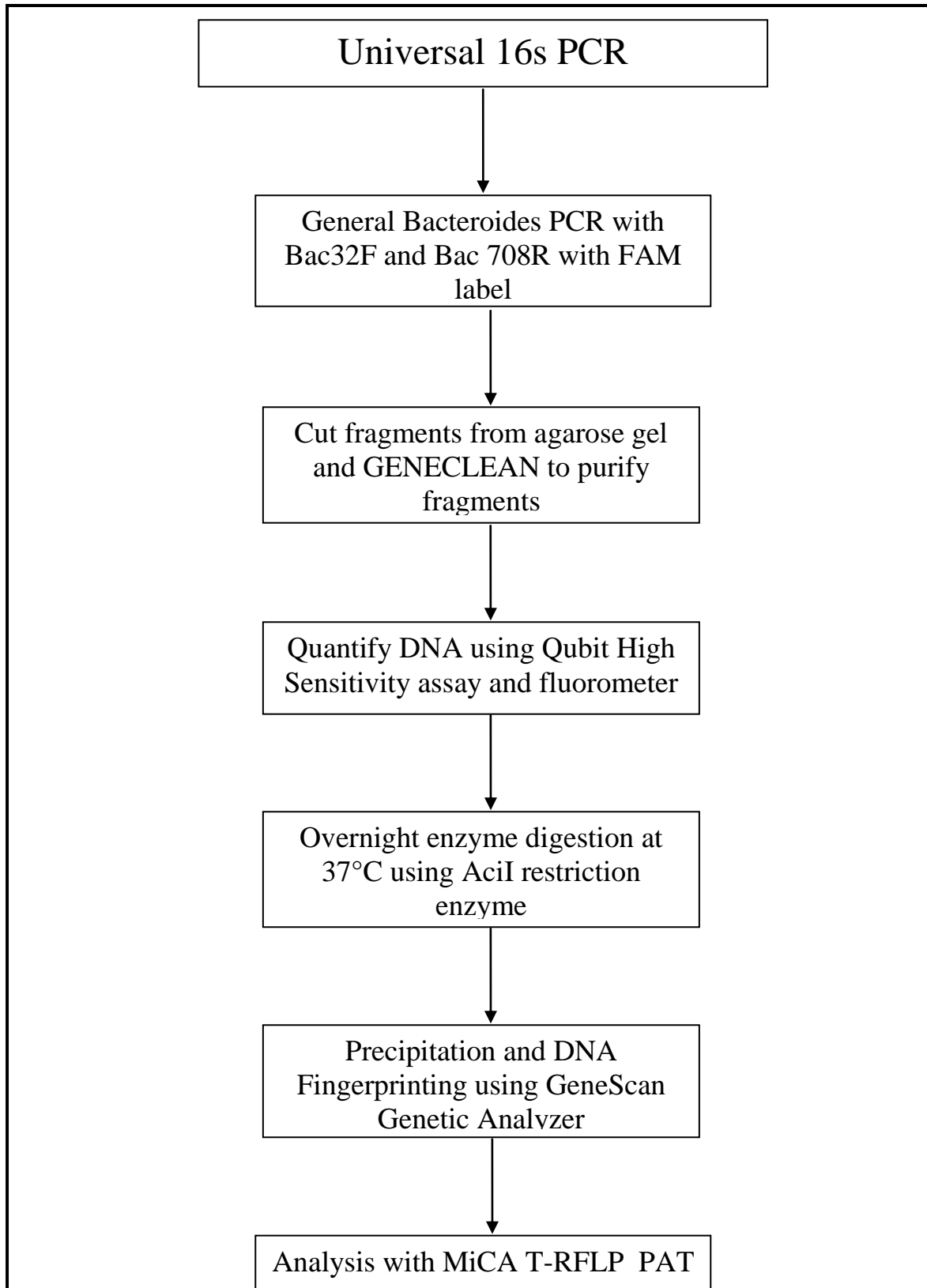


Figure 3: A flow chart outlining the T-RFLP DNA methods used.

## RESULTS AND DISCUSSION

### **Physical parameters, nutrients, and chlorophyll *a***

The physical parameters collected during field sampling via the YSI 6920 multi-parameter water quality sonde are provided for each station and month in Table 4. If there was at least one-half inch of rain within the 48-hour period before sampling, then the sample has been also marked as a rain event in Table 4. Kelsey et al. (2004) cited the need for a 48-hour rainfall measurement to be included any time fecal coliform concentrations are being researched (2004). While temperature, pH, and orthophosphate values generally stayed constant among stations in any given month, salinity, conductivity, dissolved oxygen, turbidity, nitrate/nitrite and chlorophyll *a* values varied month-to-month and station-to-station. FOY, PC-BDDS, and WC-MB had consistently high salinity and conductivity measurements, BC-NBU and HW-DT has consistently low measurements, and the remaining stations were variable in salinity and conductivity values between months. Salinity at stations BC-SC, FC-17, HW-GP, and NB-GLR historically are affected more by rainfall entering the system and by what the tides are doing at the time of sampling than the other stations.

Dissolved oxygen values were fairly consistent among stations during any given month, though there is a noticeable seasonal difference. The amount of dissolved oxygen in the water was higher during the winter months than it was during the summer months for all nine stations. This result is consistent with what was expected for two reasons. Cold water holds more oxygen than warm water, so the water usually has a higher concentration of dissolved oxygen in the colder months of the year. Bacteria also grow better in warmer environments than they do in colder ones. Any bacteria present in the system will grow faster and use more of the available dissolved oxygen in the system during the warmer months.

Table 4: The physical parameters, nutrients, and chlorophyll *a* concentrations for each month sampled at each of the nine stations.

Station	Date	Rain	Temperature	Conductivity	Salinity	Dissolved Oxygen
<b>BC-NBU</b>	December 5, 2005		17.8	269	0.1	7.2
	January 4, 2006	Rain	15.1	236	0.1	8.6
	February 27, 2006	Rain	11.6	238	0.1	10.4
	June 13, 2006	Rain	21.8	227	0.1	6.6
	July 10, 2006		21.1	285	0.1	7.1
	August 29, 2006		24.4	251	0.1	6.7
<b>BC-SB</b>	December 5, 2005		15.2	41385	26.7	7.9
	January 4, 2006	Rain	13.0	38026	24.1	8.9
	February 27, 2006	Rain	7.7	17324	10.1	10.3
	June 13, 2006	Rain	22.4	3005	1.6	5.1
	July 10, 2006		23.6	7966	4.4	5.2
	August 29, 2006		30.6	37139	23.4	4.8
<b>FC-17</b>	December 6, 2005		12.8	15187	9.2	9.4
	January 4, 2006	Rain	12.8	36655	22.9	8.5
	February 28, 2006	Rain	10.0	23337	14	9.6
	June 12, 2006		22.2	47600	2.6	4.1
	July 11, 2006		25.9	40689	26.4	2.6
	August 23, 2006	Rain	25.6	17800	10.4	3.9
<b>FOY</b>	December 6, 2005		12.8	33400	20.8	9.7
	January 4, 2006	Rain	12.9	44777	28.4	8.9
	February 28, 2006	Rain	9.6	34602	21.5	9.3
	June 12, 2006		23.7	30952	19.2	3.3
	July 11, 2006		27.3	47570	31	3.1
	August 23, 2006	Rain	26.6	42495	27.3	3.2
<b>HW-DT</b>	December 6, 2005		12.1	646	0.3	8.0
	January 3, 2006	Rain	13.5	392	0.2	9.0
	February 16, 2006		11.8	294	0.1	10.0
	June 15, 2006	Rain	23.9	536	0.3	8.1
	July 26, 2006	Rain	25.5	280	0.1	6.3
	August 28, 2006		27.9	7615	4.2	8.4
<b>HW-GP</b>	December 6, 2005		12.1	10696	6.1	7.9
	January 3, 2006	Rain	12.8	12500	7.0	9.5
	February 16, 2006		11.0	3392	1.8	9.7
	June 15, 2006	Rain	24.0	2752	1.4	6.0
	July 26, 2006	Rain	26.7	1138	0.6	5.7
	August 28, 2006		29.6	41663	26.2	5.0
<b>NB-GLR</b>	December 2, 2005		12.5	35371	22.2	7.7
	January 5, 2006	Rain	12.7	33000	21.0	9.0
	February 17, 2006		14.2	6211	3.4	9.6
	June 26, 2006		26.4	20235	11.9	4.4
	July 27, 2006	Rain	27.9	5892	3.7	6.2
	August 21, 2006		26.1	2863	1.5	6.9
<b>PC-BDDS</b>	January 24, 2007	Rain	28.8	54239	35.8	5.2
	February 20, 2007		25.3	53737	35.4	3.9
	March 22, 2007		14.1	46275	30.1	7.4
	April 17, 2007	Rain	16.5	50695	33.3	9.0
	May 31, 2007		8.4	51706	33.6	9.4
	June 18, 2007		10.5	41899	27.0	9.3
<b>WC-MB</b>	December 2, 2005		14.5	46969	30.6	7.9
	January 5, 2006	Rain	13.3	46632	30.3	9.2
	February 17, 2006		12.6	42611	27.4	10.8
	June 26, 2006		28.2	40724	26.0	5.7
	July 27, 2006	Rain	30.1	49220	32.0	5.7
	August 21, 2006		29.1	49361	32.1	4.0

Table 4 cont.

Station	pH	Turbidity	Nitrate/Nitrite	Orthophosphate	Chlorophyll a
BC-NBU	7.1	2	60.9	3.7	0.2
	7.0	10	68.9	3.2	1.5
	7.1	10	88.5	1.9	0.6
	7.1	3	63.3	2.9	0.5
	7.0	5	63.7	0.0	0.8
	6.9	7	40.5	0.2	1.1
BC-SB	7.7	2	37.1	16.3	1.8
	7.4	4	14.2	7.9	1.3
	7.6	5	35.4	7.0	0.3
	6.8	10	83.5	11.2	7.0
	7.1	7	2.1	6.1	39.7
	7.4	4	15.2	13.6	7.6
FC-17	7.7	2	56.2	12.6	1.2
	7.8	5	72.6	10.1	1.6
	7.6	5	15.4	6.0	2.4
	7.5	50	39.9	11.8	6.3
	7.6	7	132.8	6.3	0.2
	7.2	22	75.9	12.6	9.0
FOY	8.0	1	86.1	11.6	1.1
	8.0	3	22.7	6.9	1.9
	7.9	6	16.4	5.7	1.6
	7.4	15	105.1	13.9	6.2
	7.7	6	46.4	16.7	0.2
	7.5	7	44.0	13.3	8.8
HW-DT	7.8	6	101.6	23.2	1.4
	7.8	39	38.6	10.6	5.6
	7.4	11	126.9	7.2	1.3
	7.4	14	102.1	8.2	4.5
	7.3	10	77.9	11.6	6.3
	7.4	8	2.5	8.6	3.7
HW-GP	7.6	8	23.3	10.4	4.1
	7.9	12	12.5	11.7	10.1
	7.4	11	93.0	9.9	1.3
	7.4	15	69.6	15.4	17.8
	7.5	24	86.8	10.6	23.7
	7.5	8	11.9	9.8	15.4
NB-GLR	7.8	3	302.9	19.7	1.1
	7.7	4	47.6	10.1	2.4
	7.3	9	160.5	14.2	24.8
	7.3	13	22.3	8.5	9.7
	7.2	8	37.3	13.1	31.3
	7.7	15	68.5	38.1	46.4
PC-BDDS	7.7	1	7.3	8.2	0.3
	7.7	0	10.4	11.7	0.6
	7.9	8	7.6	6.0	2.2
	8.6	4	6.8	8.1	3.2
	8.1	11	15.0	10.1	0.2
	7.9	11	3.9	10.7	9.4
WC-MB	8.1	3	30.2	12.9	0.9
	8.0	2	19.6	7.1	2.6
	8.1	5	56.9	12.8	2.1
	7.7	10	5.7	11.2	1.1
	7.7	3	3.1	11.0	10.6
	7.4	12	21.5	20.3	3.5

Turbidity measurements were highly variable among stations and months of sampling. Highest turbidities were found at the Futch Creek (FC-17 and FOY) and Howe Creek (HW-DT and HW-GP) stations. These stations drain the uppermost areas of these two creeks, and have little more than two or three meters of water at the peak of high tide, so high turbidity in these shallow, often runoff influenced areas is to be expected. Otherwise, turbidity levels were fairly low during the samplings, with only a handful of values reaching 10 NTUs or above.

Orthophosphate levels were comparably low for all stations and months of sampling, with a couple of exceptions. Orthophosphate concentrations showed isolated jumps at HW-DT in December 2005 and NB-GLR and WC-MB in August 2006. BC-NBU had the lowest orthophosphate concentrations for every month of sampling.

Unlike orthophosphate, nitrate/nitrite levels were highly variable throughout the sampling. The highest of these nitrogen concentrations were found in the two Howe Creek stations, with HW-DT having a level of over 100 µg/L in half of the samples taken. PC-BDDS and WC-MB generally had the lowest nitrate/nitrite levels. The highest nitrate/nitrite concentration was recorded at NB-GLR in December 2005, with a level of 302.9 µg/L

The majority of the chlorophyll *a* values were low, with the exception of certain stations. The chlorophyll *a* values were consistently highest at NB-GLR. Other elevated chlorophyll *a* values were seen at BC-SB and HW-GP during summer months.

### **Fecal Coliform Bacteria Counts**

The fecal coliform bacteria counts for each station and month of sampling are provided in Table 5. A blank entry in the table means that no samples were taken for that month. The counts are an average of two incubated filters and are reported as the number of CFUs per 100mL of water. Fecal coliform bacteria were found to be present in every sample taken, with the



Table 5: Monthly fecal coliform bacteria counts at each station. Bolded entries are samples that were taken during rain events.

Month	BC-NBU	BC-SB	FC-17	FOY	HW-DT	HW-GP	NB-GLR	PC-BDDS	WC-MB
Dec-05	98	128	380	96	365	305	475		7
Jan-06	<b>48</b>	<b>56</b>	<b>91</b>	<b>11</b>	<b>41</b>	<b>86</b>	<b>113</b>		<b>7</b>
Feb-06	<b>60</b>	<b>127</b>	<b>123</b>	<b>35</b>	65	221	>300		1
Jun-06	<b>310</b>	<b>740</b>	1405	129	<b>213</b>	<b>201</b>	655		3
Jul-06	18	41	270	19	<b>3025</b>	<b>4075</b>	<b>272</b>		<b>1</b>
Aug-06	207	116	<b>1505</b>	<b>228</b>	173	87	615		13
Jan-07								<b>1040</b>	
Feb-07								29	
Mar-07								114	
Apr-07								<b>42</b>	
May-07								162	
Jun-07								72	
Min	18	41	91	11	41	86	113	29	1
Max	310	740	1505	228	3025	4075	655	1040	13
Geomean	82.96	121.42	366.62	52.38	218.37	272.90	358.01	109.08	3.52

exception of WC-MB for the month of July 2006. State standard MFC guidelines require a reported value of <1 for samples that have a count of 0. This value is reported as 0.5 in Table 5 and rounded up to 1. During the month of December 2005, four of the eight stations sampled had a fecal coliform level that exceeded the state standard of 200 CFUs/100mL, with NB-GLR exceeding the single-sample standard of 400 CFUs/100mL. All four of the stations that had reported counts above the standard, FC-17, HW-DT, HW-GP, and NB-GLR, have been plagued by high fecal coliform bacteria levels throughout the course of the New Hanover County Tidal Creeks Program sampling regime.

During the month of January 2006, none of the samples had an MFC count that was above the North Carolina standard. In February 2006, however, HW-GP exceeded the 200 CFU standard, and NB-GLR likely exceeded the standard as well, with an estimate of >300 for the filters that were too numerous to count. Again, NB-GLR and HW-GP were among the problem areas for high fecal coliform bacteria levels.

PC-BDDS was the only station sampled the following winter. During the month of January 2007, the fecal coliform level exceeded both the 200 CFU and 400 CFU standards, but it did not for any of the other winter or summer months of sampling for that station. The high fecal coliform count at PC-BDDS in January 2007 was the only winter sample where rain corresponded with high coliform levels.

Fecal coliform levels were consistently higher during the summer months than during the winter months. During the month of June 2006, all of the eight stations sampled exceeded the 200 CFU standard, with the exception of FOY and WC-MB. The high salinity ICW water at WC-MB kills or dilutes the majority of the fecal coliforms present as they make it to the water, so it is understandable that this station had consistently very low counts. Of the six stations in June 2006 that had very high fecal coliform counts, three of these also exceed the 400 CFU standard, namely BC-SB, FC-17, and NB-GLR. Four of these high counts also coincided with rain events, at the Bradley Creek (BC-NBU and BC-SB) and Howe Creek (HW-DT and HW-GP) stations.

With the exception of the Bradley Creek stations (BC-NBU and BC-SB), the same stations exceeded the 200 CFU standard in July 2006 as well. Howe Creek seemed to be the problem creek for the month of July 2006, with both stations of HW-DT and HW-GP exceeding the 400 CFU standard by an order of magnitude. Both of these counts also corresponded with rain events, as well as the high count at NB-GLR for that month. Howe Creek, however, had exactly the opposite in August 2006, with neither station surpassing the standard values. FC-17 and NB-GLR were a continued problem in August as well, with both stations having a count above the 400 CFU single-sample standard. BC-NBU and FOY had reported counts that were slightly

above the 200 CFU standard. Both of the high counts in the Futch Creek (FC-17 and FOY) stations coincided with rain events for the month of August 2006.

The four stations with the highest reported fecal coliform counts, FC-17, HW-DT, HW-GP and NB-GLR, also had a geometric mean that surpassed the 200 CFU standard, with FC-17 and NB-GLR reporting the highest counts overall.

## PCR

The PCR results for each station and month are provided in Table 6. The presence or absence of each target segment of DNA for each station is indicated by a yes or a no in the corresponding cell in Table 6.

The presence or absence of these target segments was determined by running the PCR results on an agarose gel like the one shown in Figure 4.

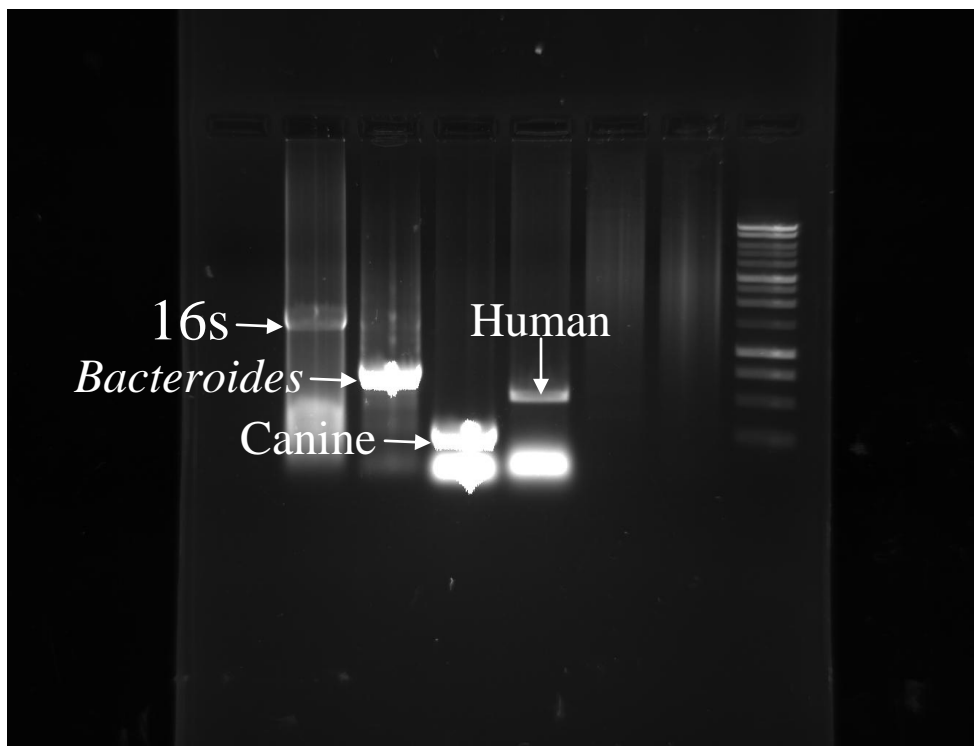


Figure 4: Agarose gel picture of PCR products.

Table 6: PCR detection results.

Station	Month	16s	Bacteroides	Canine	Ruminant	Human
BC-NBU	Dec-05	Yes	Yes	No	Yes	No
	Jan-06	Yes	Yes	No	No	No
	Feb-06	Yes	Yes	No	No	No
	Jun-06	Yes	Yes	Yes	Yes	No
	Jul-06	Yes	Yes	No	No	Yes
	Aug-06	Yes	Yes	No	No	Yes
BC-SB	Dec-05	No	No	No	No	No
	Jan-06	Yes	Yes	No	No	No
	Feb-06	Yes	Yes	Yes	No	No
	Jun-06	Yes	Yes	Yes	Yes	Yes
	Jul-06	Yes	Yes	No	No	No
	Aug-06	Yes	Yes	No	No	No
FC-17	Dec-05	Yes	Yes	No	Yes	No
	Jan-06	Yes	Yes	Yes	No	No
	Feb-06	Yes	Yes	No	No	No
	Jun-06	Yes	Yes	Yes	Yes	Yes
	Jul-06	Yes	Yes	Yes	No	No
	Aug-06	Yes	Yes	Yes	No	No
FOY	Dec-05	Yes	No	No	No	No
	Jan-06	Yes	Yes	No	No	No
	Feb-06	Yes	Yes	No	No	No
	Jun-06	Yes	Yes	Yes	Yes	No
	Jul-06	Yes	Yes	No	No	Yes
	Aug-06	Yes	Yes	No	No	No
HW-DT	Dec-05	Yes	Yes	No	Yes	No
	Jan-06	Yes	Yes	No	No	No
	Feb-06	Yes	Yes	No	No	No
	Jun-06	Yes	Yes	Yes	Yes	Yes
	Jul-06	Yes	Yes	No	No	No
	Aug-06	Yes	Yes	No	No	Yes
HW-GP	Dec-05	Yes	Yes	No	No	No
	Jan-06	Yes	Yes	No	No	No
	Feb-06	Yes	Yes	No	No	No
	Jun-06	Yes	Yes	No	Yes	No
	Jul-06	Yes	Yes	Yes	No	No
	Aug-06	Yes	Yes	No	No	No
NB-GLR	Dec-05	Yes	Yes	No	Yes	Yes
	Jan-06	Yes	No	No	No	No
	Feb-06	Yes	Yes	Yes	No	No
	Jun-06	Yes	Yes	No	Yes	No
	Jul-06	Yes	Yes	Yes	No	No
	Aug-06	Yes	Yes	No	No	No
PC-BDDS	Jan-07	Yes	Yes	No	No	Yes
	Feb-07	No	No	No	No	No
	Mar-07	Yes	Yes	No	No	No
	Apr-07	Yes	Yes	No	No	No
	May-07	No	No	No	No	No
	Jun-07	No	No	No	No	No
WC-MB	Dec-05	Yes	No	No	No	No
	Jan-06	Yes	Yes	No	No	Yes
	Feb-06	No	No	No	No	No
	Jun-06	Yes	Yes	No	Yes	No
	Jul-06	Yes	Yes	No	No	No
	Aug-06	Yes	No	No	No	No

The bands corresponding to the 16s, *Bacteroides*, canine, and human target fragments are all labeled in Figure 4. The 16s fragment has a size of 1495 basepairs, the *Bacteroides* fragment is 676 basepairs, the canine fragment is 233 basepairs, the human fragment is 525 basepairs, and the ruminant fragment is 515 basepairs.

Universal 16s PCR results were positive for the majority of the samples, with the few exceptions being BC-SB during December 2005 and January 2006, PC-BDDS in February, May and June 2007, and WC-MB during February 2006. Fecal coliform bacteria were present in every sample that was negative for 16s rDNA, so any PCR assay that looks for bacteria should be positive. The negative results could be attributed to failure in the extraction or PCR process, PCR inhibitors, or DNA concentration problems (too much or too little in the solution). Since every set of PCR reactions was run with a positive control, it is unlikely that the PCR reaction itself was the problem.

The universal *Bacteroides* PCR results were positive for the majority of the samples as well, but not all of the samples, which does not support  $H_1$  that all samples would be positive. All of the samples that were negative for 16s were also negative for *Bacteroides*, with the exception of BC-SB for the month of January 2006. Other negative *Bacteroides* samples include FOY in December 2005, NB-GLR in January 2006, and WC-MB in December 2005 and August 2006. With many of the negative results for 16s and *Bacteroides* PCR occurring during the winter months, there are two likely explanations for the negative results. The first reason could be failure in the extraction or PCR processes, or PCR inhibition. The second reason could be that bacteria grow better in the warmer months and warmer temperatures, since they are found in the guts of warm-blooded organisms. Fecal coliform bacteria were found to be present in all but one

of the samples taken, but the levels were generally lower than those found during the summer months. The negative results can most likely be attributed to a combination of both factors.

The presence of canine fecal bacteria DNA was found at seven of the nine stations via PCR, which does not support the H<sub>2</sub> prediction that a canine presence would be found at every station due to the large population of domestic animals in New Hanover County. The presence of canine DNA was found very sporadically, with the first positive being found in January 2006 at FC-17. During the month of February 2006, canine bacteria DNA was found at BC-SB and NB-GLR, which is the station downstream of a pet boarding and grooming facility. BC-NBU, BC-SB, FC-17, FOY, HW-DT, and HW-GP were all positive for canine sources during the month of June 2006. July 2006 had positives at FC-17, HW-GP, and NB-GLR. FC-17 had the only positive during August 2006. Of the 12 samples that were determined to be positive for canine fecal bacteria DNA, two-thirds of them coincided with rain events.

The presence of ruminant bacteria DNA was found during December 2005 at BC-NBU, FC-17, HW-DT and NB-GLR. The presence of ruminant bacteria DNA was also found at every station sampled during the month of June 2006. Four of the eight samples that were positive in June 2006 corresponded with stormwater runoff from rain events, namely the Bradley Creek (BC-NBU and BC-SB) and Howe Creek (HW-DT and HW-GP) stations. A horse farm is located in the area that drains to BC-SB, so runoff of horse fecal matter into Bradley Creek is likely the cause of that positive sample. The upstream stations of FC-17, FOY, HW-DT, and HW-GP all had at least one sample that was positive for ruminant bacteria DNA, which supports H<sub>3</sub>, however, there were positives at all of the other stations aside from PC-BDDS, which does not support H<sub>3</sub>. When beginning sampling at BC-SB, it was unknown that a horse was located in that area, so it was not included in H<sub>3</sub> as a likely ruminant positive area as it should have been.

New Hanover County does have a fairly large deer population, so while the findings do not support the original hypothesis, they are not surprising.

A few of the samples were found to be positive for human fecal bacteria DNA, which does not support H<sub>4</sub>. Human fecal bacteria DNA was found at NB-GLR during the month of December 2005. WC-MB was positive in January 2006. BC-SB, FC-17 and HW-DT were all positive for human fecal bacteria in June 2006. FOY was the only positive during the month of July 2006. BC-NBU and HW-DT were both positive in August 2006, and PC-BDDS was the only human positive in January 2007. Both NB-GLR and PC-BDDS have a sewer-line pump station in the area that drains to those stations, and WC-MB is a marina where several people live on houseboats.

#### **T-RFLP**

Upon completion of DNA fingerprinting, the MiCA T-RFLP PAT+ was used to determine the bacterial groups present in each sample. The PAT+ uses the resultant peak areas and fragment sizes, along with the forward and reverse primer and restriction enzyme information, to perform an analysis on the structure of the microbial community present in each sample. The resultant information makes it possible to link many of the fragment sizes to known bacterial groups and sources. A complete list of the 40 different fragment sizes found in the T-RFLP samples, along with the percentage of the sample each fragment accounted for based on peak area, can be found in Table 7. Those peaks that were identified are labeled above the fragment size in the corresponding column.

The only identified peaks that were host-specific were human-borne, however, some peaks were found that are shared between human and avian species, and some that are shared between human and ruminant sources. These peaks could have been the result of fecal bacteria pollution

Table 7: Host-specific *Bacteroides* T-RFLP peaks present in each DNA fingerprinting sample, and what percentage of the sample they account for.

Station	Month	T-RFLP Peaks												
		50	51	53	71	72	Human 102	121	124	134	141	153	157	161
BC-NBU	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06	16.69												
	Jul-06	23.14												
	Aug-06	10.98						2.47	2.37	2.23			3.17	
BC-SB	Dec-05													
	Jan-06													
	Feb-06		9.72											
	Jun-06	0.78					1.1					2.58		
	Jul-06		12.71											
	Aug-06													
FC-17	Dec-05	28.26				1.84					1.37			
	Jan-06	16.81		7.06	2.85									
	Feb-06													
	Jun-06	13.03												
	Jul-06													
	Aug-06	71.36												
FOY	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06	50.57												
	Jul-06													
	Aug-06													
HW-DT	Dec-05		2.17											
	Jan-06													
	Feb-06													
	Jun-06	14.29									2.23			
	Jul-06										4.95			
	Aug-06													
HW-GP	Dec-05										1.38			62.35
	Jan-06													
	Feb-06													
	Jun-06	26.62												
	Jul-06													
	Aug-06													
NB-GLR	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06	52.25									2.56			
	Jul-06		6.34											
	Aug-06													
PC-BDDS	Jan-07					1.17					1.26			
	Feb-07													
	Mar-07													
	Apr-07													
	May-07													
	Jun-07													
WC-MB	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06													
	Jul-06													
	Aug-06													



Table 7 cont.

		T-RFLP Peaks													
Station	Month	Human/Avian 163	Human 164	Human 165	Human 172	184	190	191	219	222	223	226	227	228	229
BC-NBU	Dec-05														
	Jan-06														
	Feb-06														
	Jun-06													68.03	
	Jul-06												34.7		
	Aug-06		3.52			2.27			2.93						41.51
BC-SB	Dec-05														
	Jan-06														
	Feb-06		2.6		3.62				3.22		2.52			26.74	39.72
	Jun-06		1.7						3.49	1.57		1.21	30.4	30.07	
	Jul-06								10.91				15.19		
	Aug-06														
FC-17	Dec-05								12.13			18.26	15.09		16.72
	Jan-06	4.89		2.28				6.0	5.3					32.54	
	Feb-06														
	Jun-06								6.91			12.83		36.84	
	Jul-06														
	Aug-06													28.64	
FOY	Dec-05														
	Jan-06														
	Feb-06														
	Jun-06								10.6				10.89		
	Jul-06				3.07										77.87
	Aug-06														
HW-DT	Dec-05				38.43										54.13
	Jan-06														
	Feb-06														
	Jun-06													38.02	
	Jul-06													31.32	
	Aug-06				19.8										
HW-GP	Dec-05				22.27								13.51	5.0	49.2
	Jan-06														
	Feb-06														
	Jun-06						3.27		7.27					39.85	
	Jul-06														
	Aug-06														
NB-GLR	Dec-05		34.47	13.04									19.1		23.42
	Jan-06														
	Feb-06		41.65	6.31	2.64						3.38			27.88	
	Jun-06								11.04					13.89	
	Jul-06				12.59				3.56					11.91	
	Aug-06														
PC-BDDS	Jan-07		3.52								3.16	1.42	24.7	55.23	
	Feb-07														
	Mar-07														
	Apr-07														
	May-07														
	Jun-07														
WC-MB	Dec-05														
	Jan-06		34.91	4.07	4.32						2.25		28.17		
	Feb-06														
	Jun-06								17.16				43.38		
	Jul-06														
	Aug-06														

Table 7 cont.

T-RFLP Peaks														
Station	Month	Human			Human			Human	Human	Human	Human	Human	Human	Human/Ruminant
		230	239	351	352	432	482	483	485	489	490	491	492	494
BC-NBU	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06												15.28	
	Jul-06											42.16		
	Aug-06		2.06		4.46								22.03	
BC-SB	Dec-05													
	Jan-06													
	Feb-06			1.09									5.68	5.09
	Jun-06	4.04		5.06								18.0		
	Jul-06	8.29					14.64			12.85		25.41		
	Aug-06													
FC-17	Dec-05						2.61					3.72		
	Jan-06				1.42	2.85		3.67				14.33		
	Feb-06													
	Jun-06	10.44		4.92								15.03		
	Jul-06													
	Aug-06													
FOY	Dec-05													
	Jan-06													
	Feb-06													
	Jun-06						7.45						20.49	
	Jul-06								3.23				3.71	12.12
	Aug-06													
HW-DT	Dec-05													5.27
	Jan-06													
	Feb-06													
	Jun-06				16.55			2.46					26.45	
	Jul-06				10.19						18.68		39.81	
	Aug-06	53.82											6.04	15.39
HW-GP	Dec-05												3.31	5.33
	Jan-06													
	Feb-06													
	Jun-06			6.27			4.48					12.23		
	Jul-06										36.0		64.0	
	Aug-06													
NB-GLR	Dec-05												4.65	5.32
	Jan-06													
	Feb-06	7.9											10.24	
	Jun-06				3.19		6.08					10.99		
	Jul-06	49.72											4.59	11.29
	Aug-06													
PC-BDDS	Jan-07											4.86		4.68
	Feb-07													
	Mar-07													
	Apr-07													
	May-07													
	Jun-07													
WC-MB	Dec-05													
	Jan-06	13.67											12.61	
	Feb-06													
	Jun-06							14.71	24.75					
	Jul-06													
	Aug-06													

from either host in those cases. While it was possible to determine that human or mixed human/animal sources did account for 13 of the 40 bacterial groups present in all of the samples, the majority of the fragment sizes were unidentifiable. Identification of these fragments can be difficult when there can be overlapping restriction fragment sizes among different species.

Several of the high percentages for human-specific peaks found in Table 7 correspond to high fecal coliform bacteria levels. For fragment size 164, both of the high percentages for NB-GLR in December 2005 and February 2006 coincide with elevated levels of fecal coliform bacteria in the water. WC-MB in January, however, had peak 164 account for a high percentage of the sample but correspond with a very low fecal coliform count.

The Howe Creek stations reported a high percentage of human fragment 172 at HW-DT in December 2005, and a high percentage of human fragment 490 at HW-GP in July 2006. Both of these high percentages were samples with high fecal coliform bacteria levels as well. Human fragment 492 was also found in high percentages at BC-NBU (August 2006), HW-DT (June and July 2006), and HW-GP (July 2006). All of these occurrences coincided with elevated fecal coliform levels, particularly the Howe Creek stations in July 2006.

Based on the peaks that were identified, host-specific group percentages were calculated for each station and month that T-RFLP was conducted. These totals are given in Table 8. According to the percentages, 6 of the 25 samples had profiles where 50% or more of the peaks were human-borne, with the two peaks present in the HW-GP July 2006 sample being 100% human-borne. If the parameters are expanded to include the number of samples where 25% or more of the peaks were human-borne, it jumps to 14 of the 25 samples, or 56%.

Many of the high percentages in the other column coincide with rain events, signaling that stormwater runoff of animal-borne fecal bacteria is the most likely cause. These were seen at

Table 8: Host-specific *Bacteroides* group percentages based on T-RFLP results.

Station	Month	Human	Human/Avian	Human/Ruminant	Other
BC-NBU	Dec-05	15.28			84.72
	Jan-06				
	Feb-06				
	Jun-06				
	Jul-06	42.16			57.84
	Aug-06	27.61			72.39
BC-SB	Dec-05				
	Jan-06				
	Feb-06	11.90		5.09	83.01
	Jun-06	20.80			79.20
	Jul-06	52.90			47.10
	Aug-06				
FC-17	Dec-05	6.33			93.67
	Jan-06	16.61	4.89		78.50
	Feb-06				
	Jun-06	15.03			84.97
	Jul-06				
	Aug-06				100.00
FOY	Dec-05				
	Jan-06				
	Feb-06				
	Jun-06	27.94			72.06
	Jul-06	9.98		12.12	77.90
	Aug-06				
HW-DT	Dec-05	38.43		5.27	56.30
	Jan-06				
	Feb-06				
	Jun-06	26.45			73.55
	Jul-06	58.49			41.51
	Aug-06	25.84		15.39	58.77
HW-GP	Dec-05	25.58		5.33	69.09
	Jan-06				
	Feb-06				
	Jun-06	16.71			83.29
	Jul-06	100.00			
	Aug-06				
NB-GLR	Dec-05	57.48			42.52
	Jan-06				
	Feb-06	50.60		10.24	39.16
	Jun-06	17.07			82.93
	Jul-06	17.18		11.29	71.53
	Aug-06				
PC-BDDS	Jan-07	8.38		4.68	86.94
	Feb-07				
	Mar-07				
	Apr-07				
	May-07				
	Jun-07				
WC-MB	Dec-05				
	Jan-06	55.91			44.09
	Feb-06				
	Jun-06	24.75			75.25
	Jul-06				
	Aug-06				

BC-SB (February, June and July 2006), FC-17 (January and August 2006), HW-DT (June and July 2006), HW-GP (June 2006), NB-GLR (July 2006), PC-BDDS (January 2007), and WC-MB (January 2006). Seven of these eleven instances corresponded with high fecal coliform bacteria levels as well, which have been linked to rainfall events (Young and Thackston 1999). Several of the high percentage totals found for human fragments also coincide with high levels of fecal coliform bacteria in the water. HW-DT (December 2005, June and July 2006), HW-GP (December 2005, July 2006), and NB-GLR (December 2005 and February 2006) all had fecal coliform levels that exceeded the state standard during the indicated months and all had a high reported percentage of human-borne fragments present in the T-RFLP profiles.

T-RFLP also resulted in a profile corresponding to each sample, with peaks of varying fragment sizes and heights representing the different bacterial groups present in the sample and their relative abundance. Of those peaks, the peaks that were identified using the MiCA T-RFLP PAT+ are labeled with their fragment size and host-specificity in Figures 5-13. The more inconspicuous peaks are unlabeled in these figures, as they are difficult to see and represent only a very small portion of the bacterial community present in the sample.

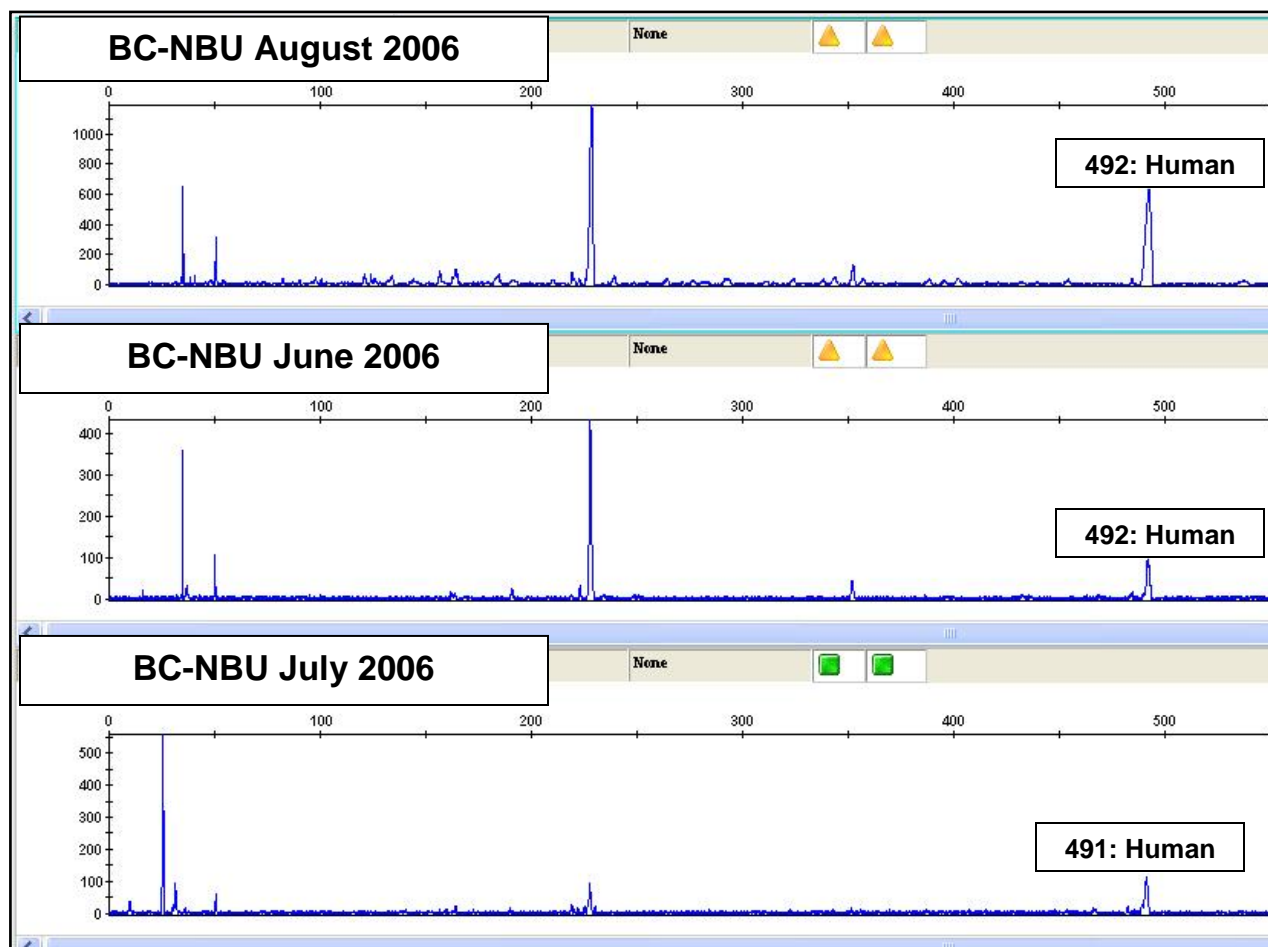


Figure 5: T-RFLP profiles from BC-NBU for the months of August (top), June (middle), and July (bottom) 2006.

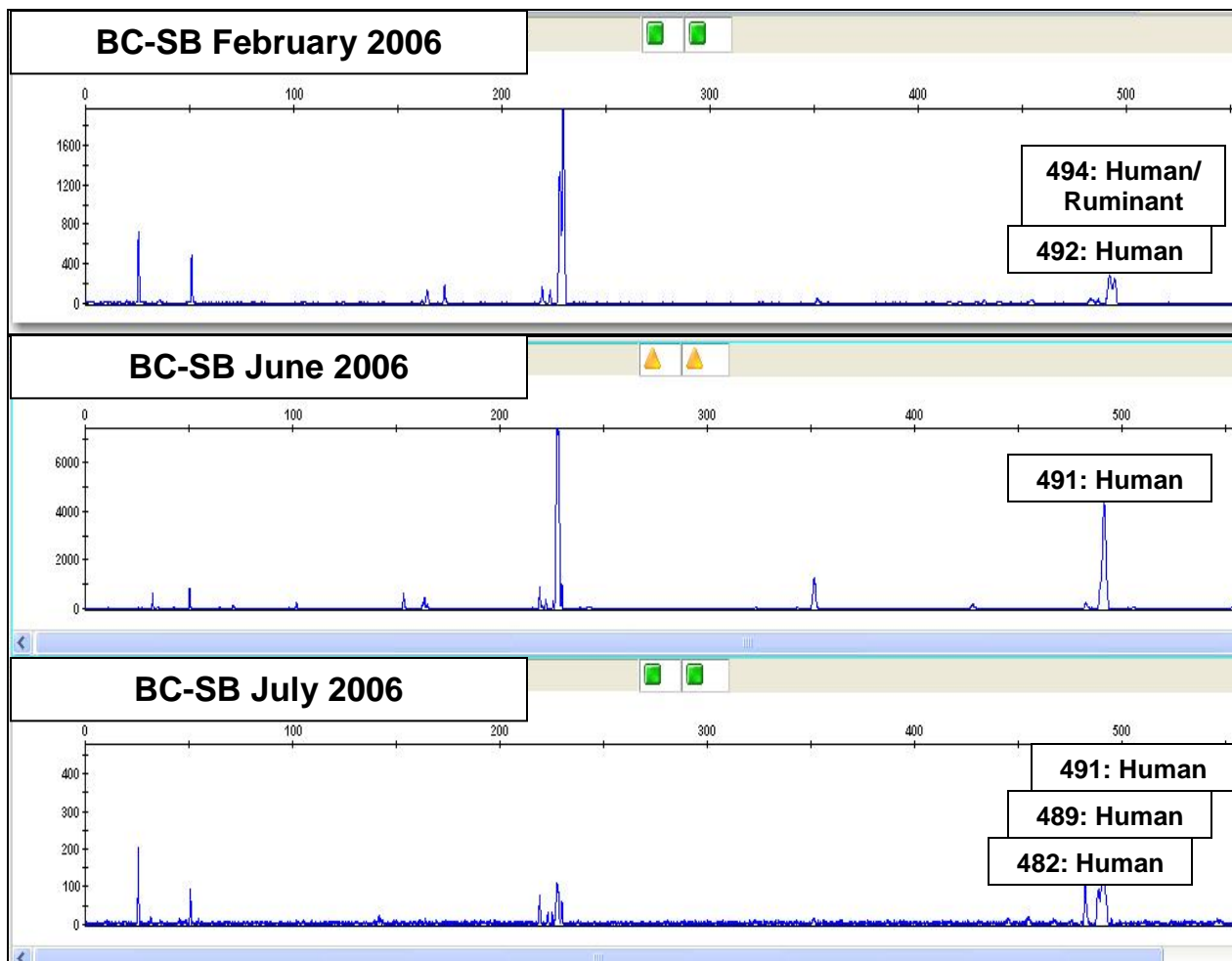


Figure 6: T-RFLP profiles from BC-SB for the months of February (top), June (middle), and July (bottom) 2006.

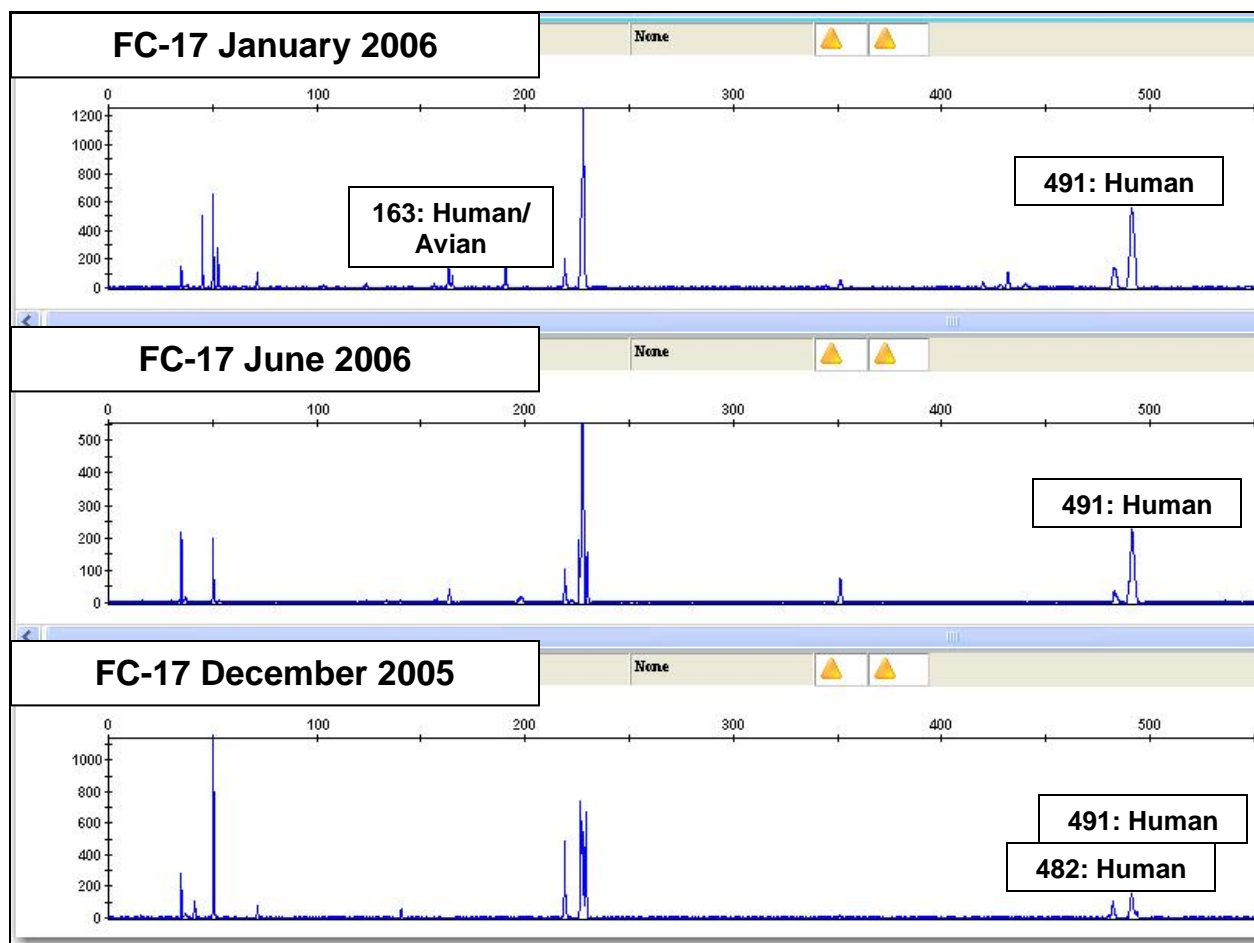


Figure 7: T-RFLP profiles from FC-17 for the months of January (top) and June (middle) 2006 and December (bottom) 2005.



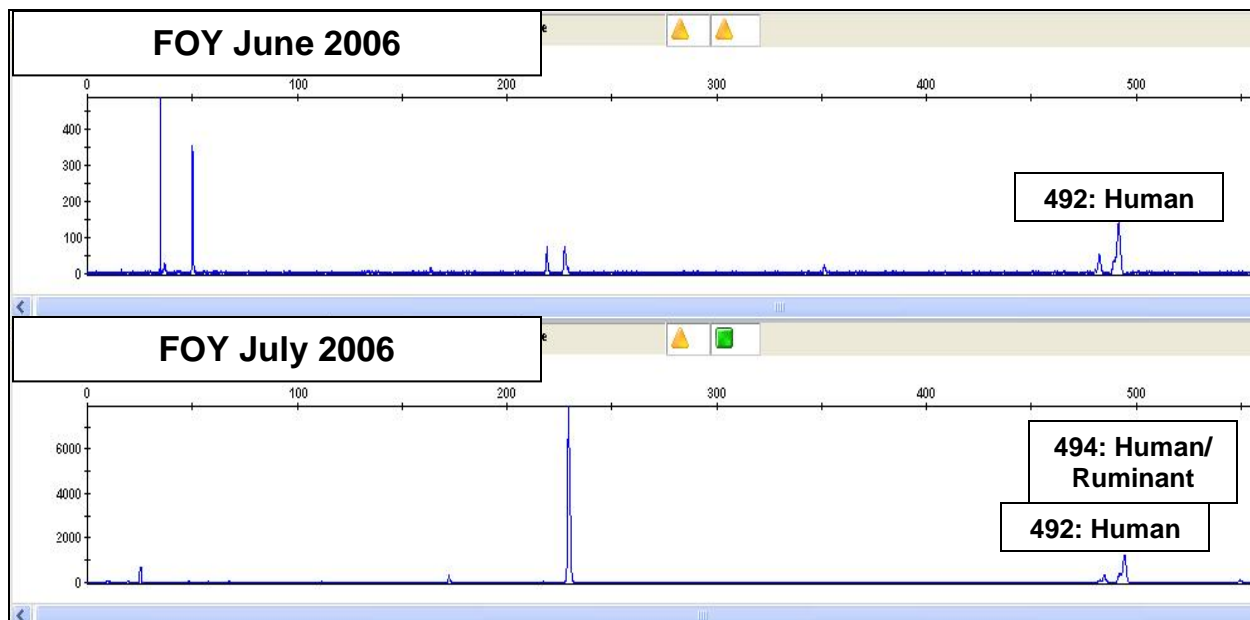


Figure 8: T-RFLP profiles from FOY for the months of June (top) and July (bottom) 2006.

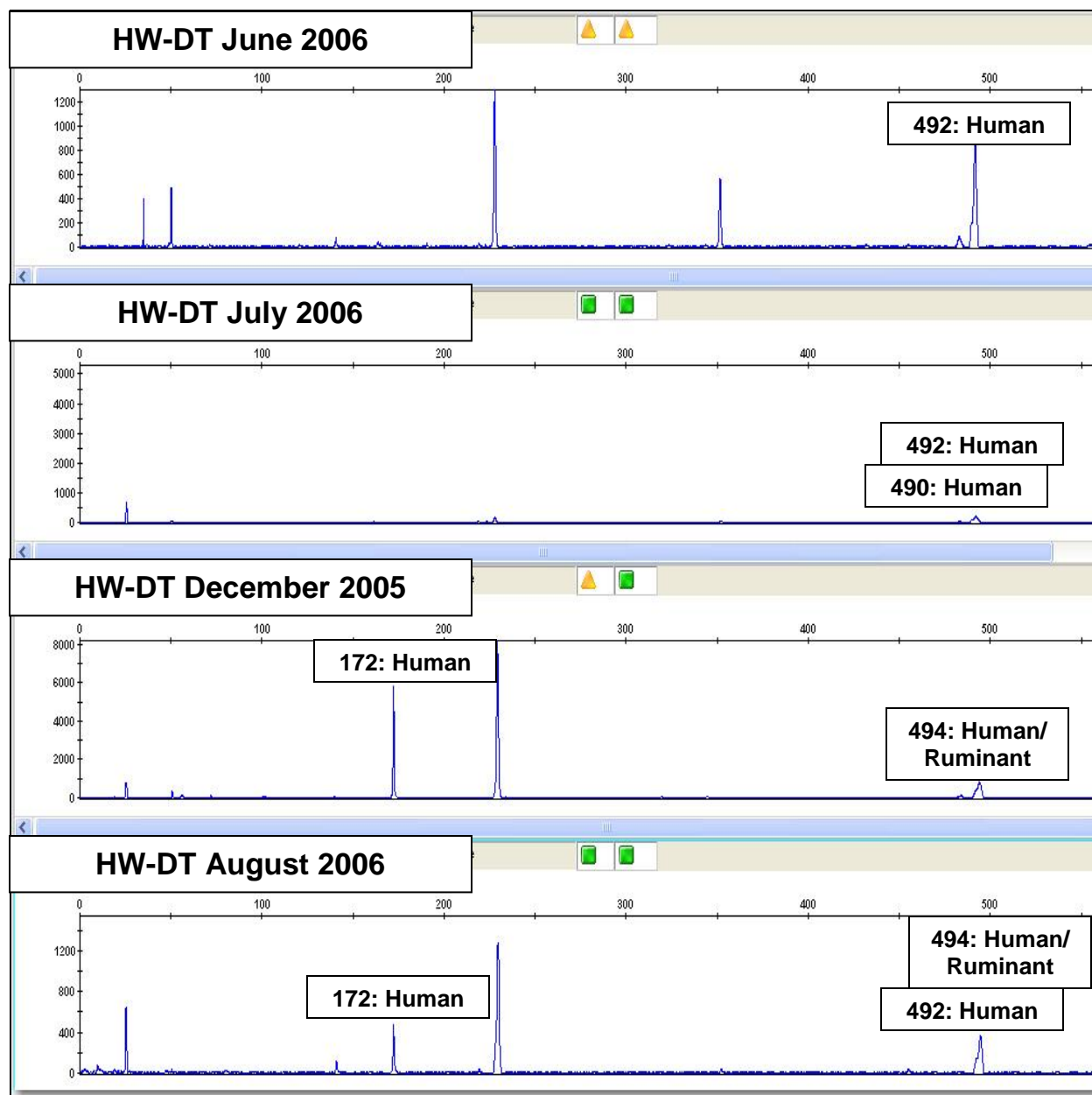


Figure 9: T-RFLP profiles from HW-DT for the months of June (top), July (second), and August (bottom) 2006 and December (third) 2005.

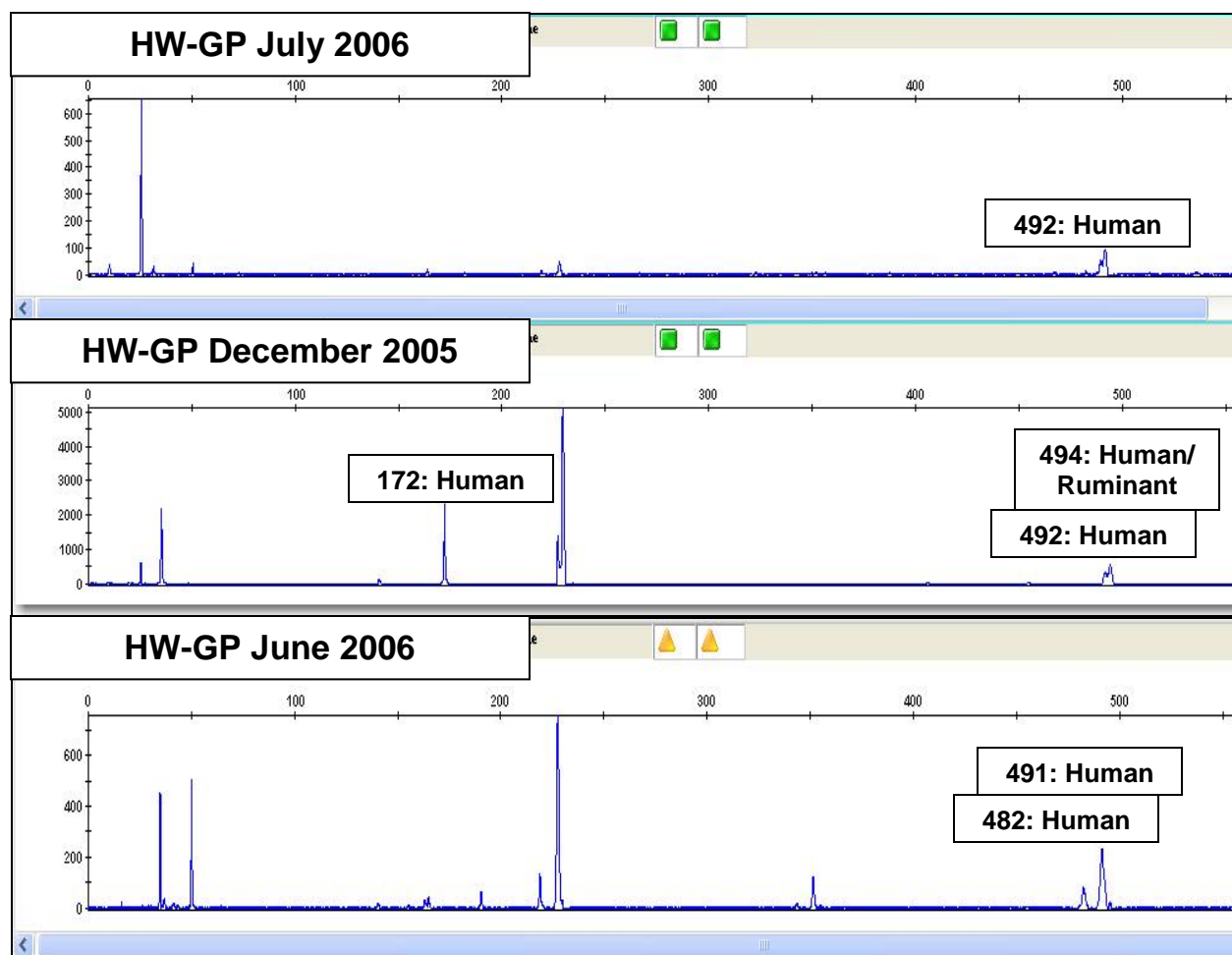


Figure 10: T-RFLP profiles from HW-GP for the months of July (top) and June (bottom) 2006 and December (middle) 2005.

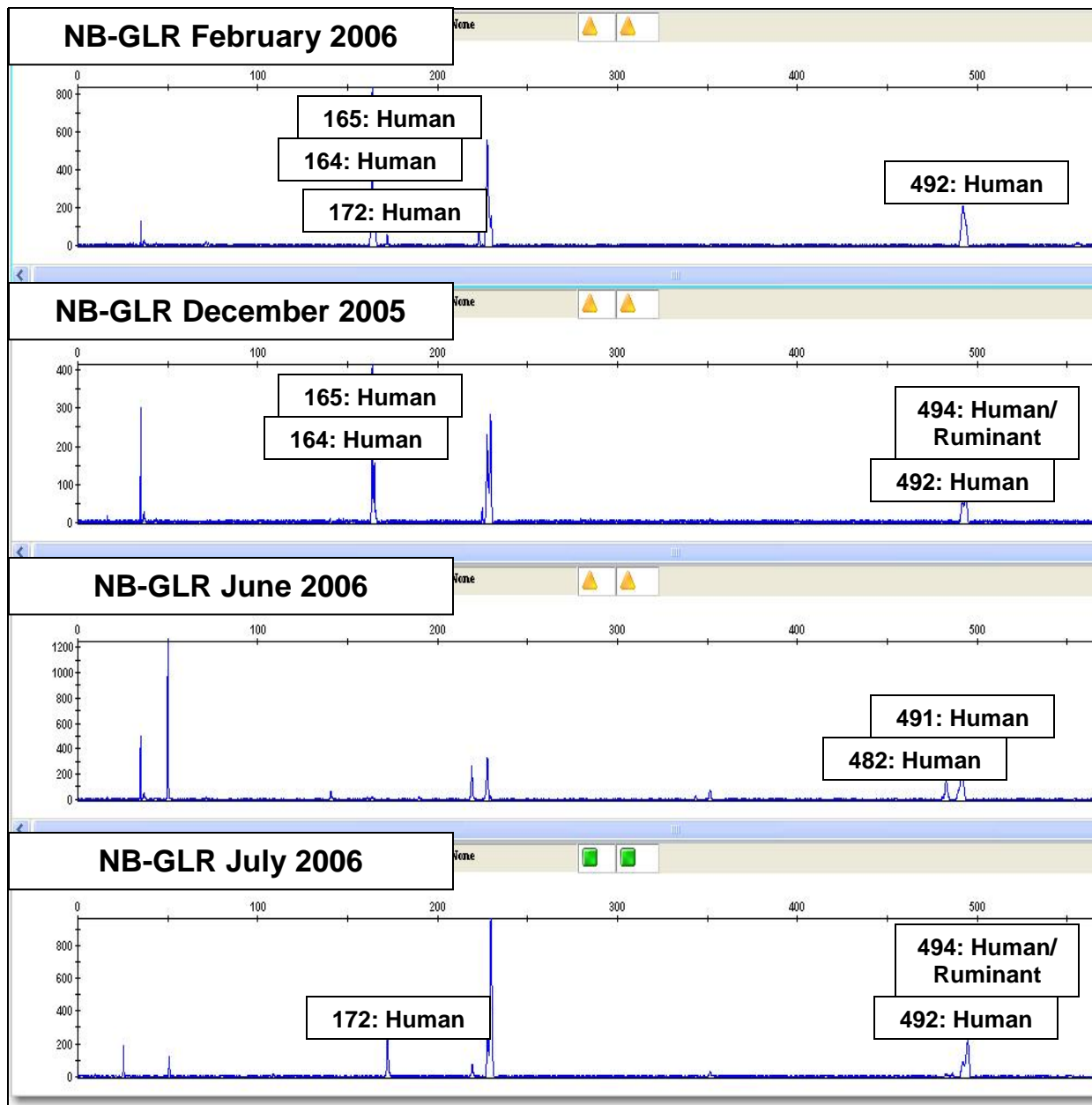


Figure 11: T-RFLP profiles from NB-GLR for the months of February (top) and June (third) and July (bottom) 2006, and December (second) 2005.

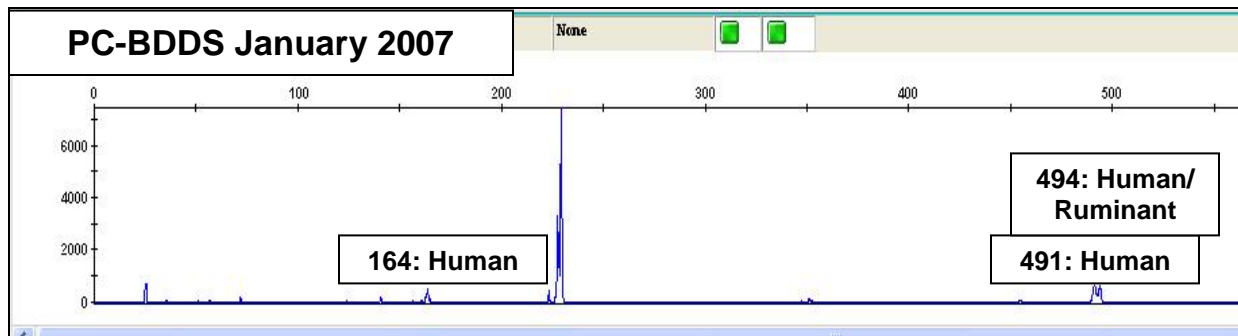


Figure 12: T-RFLP profile from PC-BDDS for the month of January 2007.

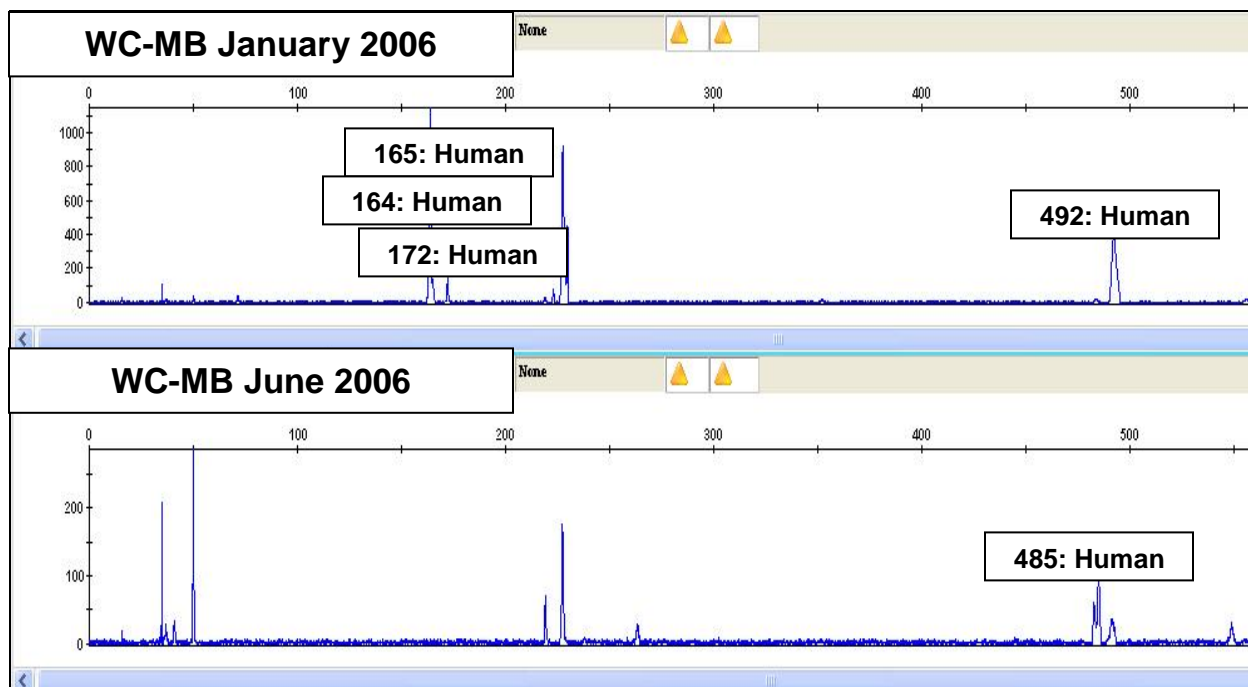


Figure 13: T-RFLP profiles from WC-MB for the months of January (top) and June (bottom) 2006.

In an effort to obtain a positive control sample to use for comparison, one sample was taken from each of four different sampling locations on Hewletts Creek immediately following a major sewer-line failure in November 2006. DNA was extracted from each of these samples and they were processed for fingerprinting as well. The resultant profiles are provided in Figure 14.

In these positive control profiles, it is possible to see the consistency in the peaks that have been identified. Human peaks 164 and 492 were found in all four of the sewage spill samples, 172 was found in three of the samples, and 165 was found in two of them. Some or all of these fragment sizes were found to be present in the experimental T-RFLP profiles. This positive control offers a good basis for comparison for the other tidal creeks.

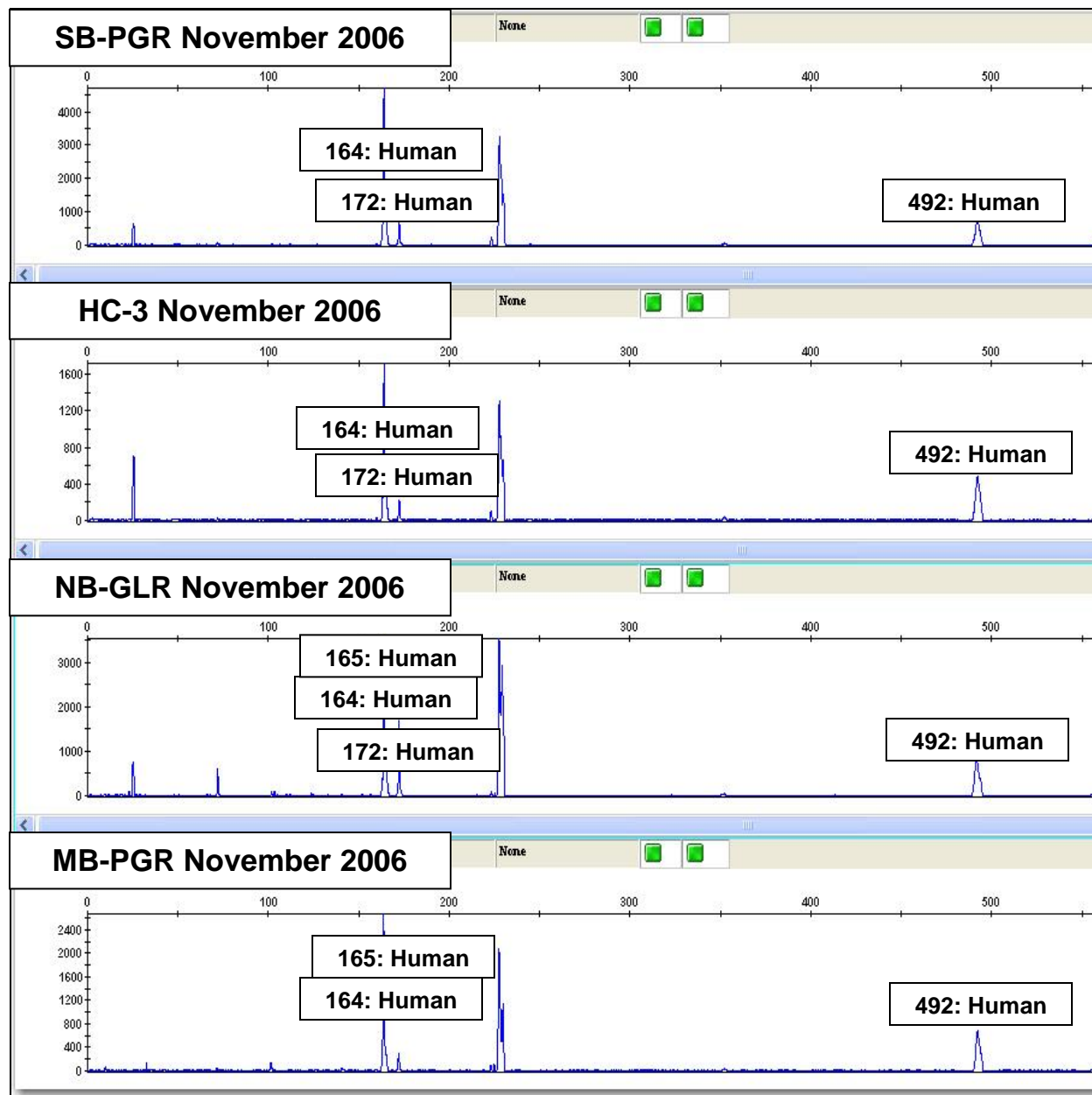


Figure 14: T-RFLP profiles from Hewlett's Creek stations SB-PGR (top), HC-3 (second), NB-GLR (third) and MB-PGR (bottom) for the November 2006 sewage spill.

A principal component analysis (PCA) comparing the physical properties and chlorophyll *a* values from Table 4 to the host-specific *Bacteroides* group percentages provided in Table 8 is provided in Figure 15. Based on a visual analysis of this PCA, the *Bacteroides* groups seem to have a positive correlation with temperature, turbidity, nitrate/nitrite, chlorophyll *a*, and a negative correlation with salinity, conductivity, dissolved oxygen and pH.

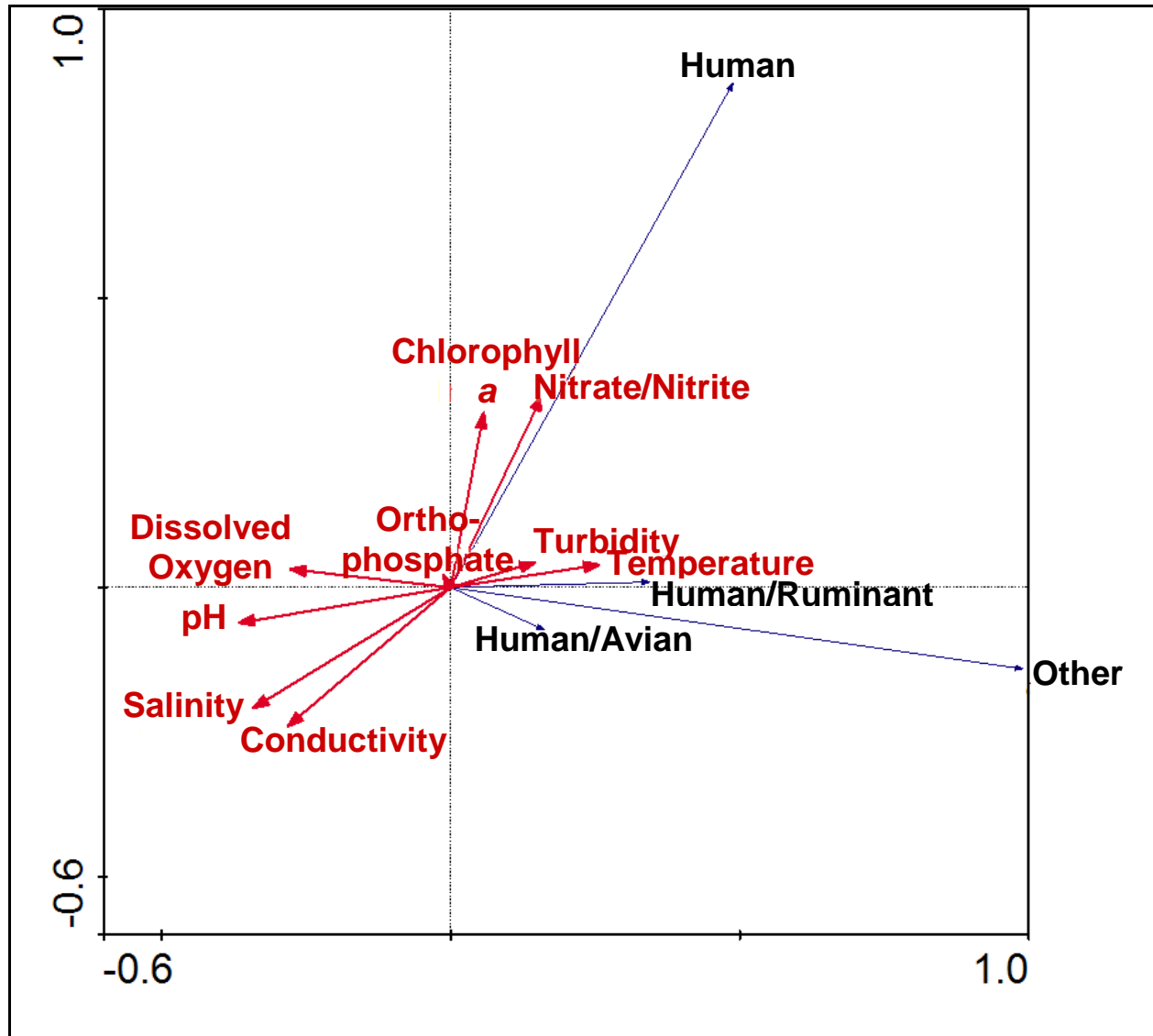


Figure 15: A principal component analysis comparing the physical properties, nutrients, and chlorophyll *a* concentrations to host-specific *Bacteroides* group percentages.

A linear regression analysis was also conducted to compare Tables 4 and 8. This analysis compared each of the physical parameters in Table 4 to the host-specific percentages in Table 8 to determine the strength and direction of the relationship between two variables, and the associated probability. The Pearson product-moment correlation coefficient ( $r$ ) indicates the strength and direction of the correlation between two variables. The higher this value is, the stronger the correlation is between the two variables being compared. A positive value indicates a positive correlation, a negative value indicates a negative correlation. The probability values are denoted as ( $P$ ). The results of this correlation analysis are provided in Table 9. These correlation coefficients confirm that all of the host-specific groups are positively correlated with temperature, turbidity, nitrate/nitrite, and chlorophyll  $a$ , with the exception of human/avian, which has a negative correlation with all but nitrate/nitrite and human/ruminant, which has a negative correlation with nitrate/nitrite and turbidity. All of the host specific groups are negatively correlated with conductivity, salinity, dissolved oxygen, and pH, with the exception again of human/avian, which is negatively correlated with all parameters.

Of these correlations, a few are statistically significant, meaning they have an associated  $P$ -value of 0.05 or less. Human-specific peaks were found to have a significant negative correlation with salinity and conductivity, and a significant positive correlation with nitrate/nitrite and chlorophyll  $a$ . Human/ruminant fragments were also found to be significantly negatively correlated with salinity. The unidentifiable other fragments were found also to have a significant negative correlation with salinity, as well as with pH and dissolved oxygen.



Table 9: Linear regression analysis results comparing physical parameters, nutrients, and chlorophyll *a* concentrations to host- specific group percentages. Presented as Pearson product-moment correlation coefficient (*r*) and probability (*P*).

Host Specificity	Statistical Value	Temperature	Conductivity	Salinity	Dissolved Oxygen	pH
Human	<i>r</i>	0.159845	-0.3477	-0.34959	-0.10808	-0.23239
	<i>P</i>	0.248266	0.009972	0.002898	0.436623	0.090834
Human/Avian	<i>r</i>	-0.12148	0.085132	0.084749	0.079492	0.093091
	<i>P</i>	0.381565	0.540496	0.542323	0.56774	0.503159
Human/Ruminant	<i>r</i>	0.153956	-0.12989	-0.1149	-0.01024	-0.08305
	<i>P</i>	0.266349	0.349216	0.0408047	0.941426	0.550479
Other	<i>r</i>	0.249328	-0.245281	-0.30912	-0.279551	-0.354476
	<i>P</i>	0.069043	0.073827	0.02294	0.040637	0.008541

Table 9 cont.

Host Specificity	Statistical Value	Turbidity	Nitrate/Nitrite	Orthophosphate	chlorophyll <i>a</i>
Human	<i>r</i>	0.110066	0.367798	0.011844	0.291578
	<i>P</i>	0.428182	0.006217	0.932257	0.032416
Human/Avian	<i>r</i>	-0.06403	0.053009	-0.011607	-0.06908
	<i>P</i>	0.645517	0.703437	0.933612	0.619641
Human/Ruminant	<i>r</i>	-0.089649	-0.014647	0.131269	0.138359
	<i>P</i>	0.519144	0.916283	0.344067	0.318403
Other	<i>r</i>	0.139598	0.110086	0.016613	0.01381
	<i>P</i>	0.314045	0.4281	0.905092	0.921047

## CONCLUSIONS

More than 85% of the samples were positive for general *Bacteroides* DNA. The original hypothesis states that general *Bacteroides* DNA would be found at all of the nine stations, which was the case. However, not all stations were positive for *Bacteroides* during every month of sampling, despite being positive for fecal coliform bacteria. It is likely that some mishandling may have occurred during the extraction or PCR processes to affect the viability of the DNA, or PCR inhibitors may have been present in the solution.

The presence of canine fecal bacteria DNA was found at seven of the nine stations, but was not nearly as prevalent as predicted. Only 12 of the 54 samples were positive for canine bacteria, which is less than 23%. The observed lack of a canine presence is a positive sign that the City of Wilmington and the town of Wrightsville Beach, both in New Hanover County, have well-working management systems in place to encourage pet owners to clean-up after their pets. Developing such a program on a county level as well would be beneficial.

More than 18% of the samples were positive for human fecal bacteria DNA, which vastly exceeds the predicted 0% presence. 20% of these positive samples were from Futch Creek (FC-17 and FOY), an area that still has residents on individual septic systems. Further sample processing through T-RFLP identified human-specific fragment sizes and their relative abundance in these positive samples. Principal component analysis revealed that human fecal bacteria DNA had a positive correlation with temperature, turbidity, nitrate/nitrite, and chlorophyll *a* concentrations, and a negative correlation with conductivity, salinity, and pH. Fecal bacteria live in the guts of warm-blooded animals and grow well in comparable conditions, which explains a positive correlation with temperature. Fecal coliforms are also very salinity sensitive, which explains why there was a negative correlation with salinity and conductivity. Of

the reported *P*-values, the positive correlation with nitrate/nitrite and chlorophyll *a*, and the negative correlation with salinity and conductivity, were all found to be statistically significant.

Ruminant fecal bacteria DNA was found in the predicted four upstream areas, as well as four of the other five creeks sampled. Like the canine DNA, 12 of the 54 samples were positive for ruminant sources of fecal bacteria pollution. Of the 12 positives, four of them were following a June 2006 rain event. Principal component and regression analyses showed that the human/ruminant specific fecal bacteria behaved the same as the human alone, with a positive correlation to chlorophyll *a*, nitrate/nitrite, temperature, and turbidity, and a negative correlation to the other physical parameters, particularly conductivity and salinity. Of these, only a negative correlation with salinity was found to be statistically significant.

The one fragment size identified as human/avian behaved almost exactly the opposite of the other host-specific fragments, with a negative correlation to temperature, turbidity, orthophosphate, and chlorophyll *a* and a positive correlation to the other physical parameters. None of the values were found to be significant.

The presence of human fecal bacteria pollution is of tremendous concern. New Hanover County has a well-known history of sewer line problems. Old lines are incapable of accommodating all of the new connections and lines have failed on a number of occasions. Evidence of human fecal bacteria pollution is indicative of further leaks in the sewer system, septic system failures (where they are present), or general persistence of human-specific *Bacteroides* bacteria in the creeks themselves, as has been seen in past research (Conboy and Goss 2001). Background research is needed to help identify the unknown fragment sizes and make further conclusions on a course of action for watershed management.

## REFERENCES

- APHA, AWWA, and WEF. Standard Methods for the Examination of Water and Wastewater. 20<sup>th</sup> Edition. 1998
- Bernhard, A.E. and K.G. Field. 2000a. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Applied and Environmental Microbiology* **66**:1587-1594.
- Bernhard, A.E., and K.G. Field. 2000b. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. *Applied and Environmental Microbiology* **66**:4571-4574.
- Burkhardt, W, K.R. Calci, W.D. Watkins, S.R. Rippey, and S.J. Chirtel. 1999. Inactivation of indicator microorganisms in estuarine waters. *Water Research* **34**:2207-2214.
- Canale, R. P., M. T. Auer, E. M. Owens, T. M. Heidtke, and S. W. Effler. 1993. Modeling fecal coliform bacteria—II. Model development and application. *Water Research* **27**:703-714.
- Conboy, M. J., and M. J. Goss. 2001. Identification of an assemblage of indicator organisms to assess timing and source of bacterial contamination in groundwater. *Water, Air, and Soil Pollution* **129**:101-118.
- Dame, R., M. Alber, D. Allen, A. Chalmers, C. Gardner, B. Gilman, B. Kjerfve, A. Lewitus, M. Mallin, C. Montague, J. Pinckney, and N. Smith. 2000. Estuaries of the south Atlantic coast of North America: Their geographical signatures. *Estuaries* **23**:793-819.
- Dick, L.K., M.T. Simonich, and K.G. Field. 2005. Microplate subtractive hybridization to enrich for Bacteroidales genetic markers for fecal source identification. *Applied and Environmental Microbiology* **71**:3179-3183.
- Entry, J. A., and N. Farmer. 2001. Ground water quality: Movement of coliform bacteria and nutrients in ground water flowing through basalt and sand aquifers. *Journal of Environmental Quality* **30**:1533-1539.
- Field, K.G., A.E. Bernhard, and T.J. Brodeur. 2003. Molecular approaches to microbiological monitoring: fecal source detection. *Environmental Monitoring and Assessment* **81**:313-326.
- Gannon, J.J., and M.K. Busse. 1989. *E. Coli* and enterococci levels in urban stormwater, river water and chlorinated treatment plant effluent. *Water Research* **23**:1167-1176.
- Griffin, D.W., C.J. Gibson, E.K. Lipp, K. Riley, J.H. Paul, and J.B. Rose. 1999. Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. *Applied and Environmental Microbiology* **65**:4118-4125.
- Guan, S., R. Xu, S. Chen, J. Odumeru, and C. Gyles. 2002. Development of a procedure for

- discriminating among *Escherichia coli* isolates from human and animal sources. *Applied Environmental Microbiology* **68**:2690-2698.
- Holland, A.F., D.M. Sanger, C.P. Gawle, S.B. Lerberg, M.S. Santiago, G.H.M. Riekerk, L.E. Zimmerman, and G.I. Scott. 2004. Linkages between tidal creek ecosystems and the landscape and demographic attributes of their watersheds. *Journal of Experimental Marine Biology and Ecology* **298**:151-178.
- Jamieson, R. C., R. J. Gordon, S. C. Tatttrie, and G. W. Stratton. 2003. Sources and persistence of fecal coliform bacteria in a rural watershed. *Water Quality Research Journal of Canada* **38**:33-47.
- Kelsey, H., D. E. Porter, G. Scott, M. Neet, and D. White. 2004. Using geographic information systems and regression analysis to evaluate relationships between land use and fecal coliform bacterial pollution. *Journal of Experimental Marine Biology and Ecology* **298**:197-209.
- Kim, D-H., Sack, W. A., and D. Gera. 1996. Measurement of fecal coliform bacteria from domestic waste. *Toxicological and Environmental Chemistry* **56**:249-257.
- Klug, W.S., and M.R. Cummings. 1997. *Concepts of Genetics*. Fifth Edition. New Jersey: Prentice Hall.
- Mallin, M. A., E. C. Esham, K. E. Williams and J. E. Nearhoof. 1999. Tidal stage variability of fecal coliform and chlorophyll *a* concentrations in coastal creeks. *Marine Pollution Bulletin* **38**:414-422.
- Mallin, M. A. 2000. Impacts of industrial animal production on rivers and estuaries. *American Scientist* **88**:2-13.
- Mallin, M. A., K. E. Williams, E. C. Esham, and R. P. Lowe. 2000. Effect of human development on bacteriological water quality in coastal watersheds. *Ecological Applications* **10**:1047-1056.
- Mallin, M. A., S. H. Ensign, M. R. McIver, G. C. Shank, and P. K. Fowler. 2001. Demographic, landscape, and meteorological factors controlling the microbial pollution of coastal waters. *Hydrobiologia* **460**:185-193.
- Mallin, M. A., M. H. Posey, M. R. McIver, D. C. Parsons, S. H. Ensign, and T. D. Alphin. 2002. Impacts and recovery from multiple hurricanes in a Piedmont-Coastal Plain river system. *BioScience* **52**:999-1010.
- Mallin, M. A., and L. B. Cahoon. 2003. Industrialized animal production—A major source of nutrient and microbial pollution to aquatic ecosystems. *Population and Environment* **24**:369-385.
- Mallin, M.A. and A. J. Lewitus. 2004. The importance of tidal creek ecosystems. *Journal of*

Experimental Marine Biology and Ecology **298**:145-149.

- Marsalek, J, and Q. Rochfort. 2004. Urban wet-weather flows: Sources of fecal contamination impacting on recreational waters and threatening drinking-water sources. *Journal of Toxicology and Environmental Health, Part A* **67**:1765-1777.
- McLellan, S., and E. Jensen. 2003. Lake Michigan beaches: Urban stormwater and water quality advisories. *LakeLine* **23**:27-29.
- North Carolina Department of Environment and Natural Resources (NCDENR). 2005. Cape Fear River Basinwide Water Quality Plan, October 2005. NCDENR, Division of Water Quality, Planning Section, Raleigh, N.C.
- Nobles, R.E., P. Brown, J.B. Rose, and E.K. Lipp. 2000. The investigation and analysis of swimming-associated illness using the fecal indicator enterococcus in southern Florida's marine waters. *Florida Journal of Environmental Health* **169**:15-19.
- Olyphant, G. 2003. Beach closure decisions: The case for developing and using predictive models. *LakeLine* **23**:19-22.
- Parveen, S., R.L. Murphree, L. Edmiston, C.W. Kaspar, K.M. Portier, and M.L. Tamplin. 1997. Association of multiple-antibiotic-resistance profiles with point and non-point sources of *Escherichia coli* in Apalachicola Bay. *Applied Environmental Microbiology* **63**:2607-2612.
- Suzuki, M.T., and S.J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16s rRNA genes by PCR. *Applied and Environmental Microbiology* **62**:625-630.
- USFDA. 1995. Sanitation of shellfish growing areas. National Shellfish Sanitation Program Manual of Operations, Part 1. United States Department of Health and Human Services, Food and Drug Administration, Office of Seafood, Washington, D.C.
- Webster, L. F., B. C. Thompson, M. H. Fulton, D. E. Chestnut, R. F. Van Dolah, A. K. Leight, and G. I. Scott. 2004. Identification of sources of *Escherichia coli* in South Carolina estuaries using antibiotic resistance analysis. *Journal of Experimental Marine Biology and Ecology* **298**:179-195.
- Young, K.D. and E.L. Thackston. 1999. Housing density and bacterial loading in urban streams. *Journal of Environmental Engineering* **125**:1177-1180.